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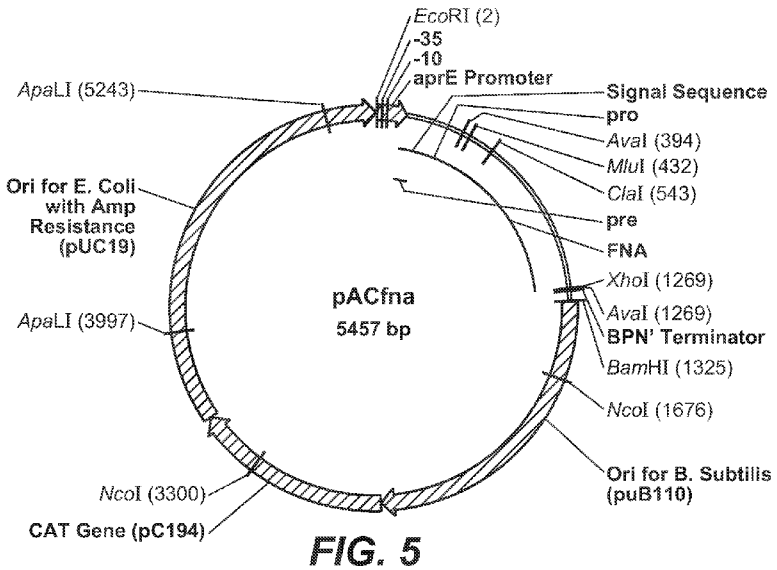

FIG. 5

(57) **Abstract**: The invention relates to modified polynucleotides encoding modified proteases, and methods for altering the production of proteases in microorganisms. In particular, the modified polynucleotides comprise one or more mutations that encode modified proteases having modifications of the pre-pro region that enhance the production of the active enzyme. The present invention further relates to methods for altering the production of proteases in microorganisms, such as *Bacillus* species.

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PROTEASES WITH MODIFIED PRE-PRO REGIONS

FIELD OF THE INVENTION

[001] This invention relates to modified polynucleotides encoding modified proteases, and methods for altering the production of proteases in microorganisms. In particular, the modified polynucleotides comprise one or more mutations that encode modified proteases having modifications of the pre-pro region that enhance the production of the active enzyme. The present invention further relates to methods for altering the production of proteases in microorganisms, such as *Bacillus* species.

BACKGROUND

[002] Proteases of bacterial origin are important industrial enzymes that are responsible for the majority of all enzyme sales, and are utilized extensively in a variety of industries, including detergents, meat tenderization, cheese-making, dehairing, baking, brewery, the production of digestive aids, and the recovery of silver from photographic film. The use of these enzymes as detergent additives stimulated their commercial development and resulted in a considerable expansion of fundamental research into these enzymes (Germano et al. *Enzyme Microb. Technol.* 32:246–251 [2003]). In addition to detergent and food additives, proteases *e.g.* alkaline proteases have substantial utilization in other industrial sectors such as leather, textile, organic synthesis, and waste water treatment (Kalisz, Adv. Biochem. Eng. Biotechnol., 36:1-65 [1988]) and (Kumar and Takagi, Biotechnol. Adv., 17:561-594 [1999]).

[003] Consequent to the high demand for these industrial enzymes, alkaline proteases with novel properties have continued to be the focus of research interest, which has led to newer protease preparations with improved catalytic efficiency and better stability towards temperature, oxidizing agents and changing usage conditions. However, the overall cost of enzyme production and downstream processing remains the major obstacle against the successful application of any technology in the enzyme industry. To this end, researchers and process engineers have used several methods to increase the yields of alkaline proteases with respect to their industrial requirements.

[004] In spite of the implementation of various approaches for increasing protease yield, including screening for hyper-producing strains, cloning and over-expressing proteases, improving fed-batch and chemostat fermentations, and optimizing fermentation technologies, there remains a need for additional means for enhancing the production of proteases.

SUMMARY OF THE INVENTION

[005] This invention provides modified polynucleotides encoding modified proteases, and methods for altering the production of proteases in microorganisms. In particular, the modified polynucleotides comprise one or more mutations that encode modified proteases having modifications of the pre-pro

region that enhance the production of the active enzyme. The present invention further relates to methods for altering the production of proteases in microorganisms, such as *Bacillus* species.

[006] In one embodiment, the present invention provides an isolated modified polynucleotide that encodes a modified full-length protease, wherein the isolated modified polynucleotide comprises a first polynucleotide that encodes the pre-pro region of the full-length protease, and that is operably linked to a second polynucleotide that encodes the mature region of the full-length protease, wherein the first polynucleotide encodes the pre-pro region of SEQ ID NO:7, which is further mutated to comprise at least one mutation that enhances the production of the protease by a host cell. Preferably, the host cell is a *Bacillus* sp. host cell *e.g.* a *Bacillus subtilis* host cell. In some embodiments, the modified full-length protease is a serine protease that is derived from a wild-type or a variant parent serine protease *e.g.* a *Bacillus subtilis*, a *Bacillus amyloliquefaciens,* a *Bacillus pumilis* or a *Bacillus licheniformis* serine protease.

[007] In another embodiment, the present invention provides an isolated modified polynucleotide that encodes a modified full-length protease, wherein the isolated modified polynucleotide comprises a first polynucleotide that encodes the pre-pro region of the full-length protease, and that is operably linked to a second polynucleotide that encodes the mature region of the full-length protease, wherein the first polynucleotide encodes the pre-pro region of SEQ ID NO:7, which is further mutated to comprise at least one mutation that enhances the production of the protease by a host cell, and the second polynucleotide encodes a protease that has at least about 65% identity to the mature protease of SEQ ID NO:9. Preferably, the second polynucleotide encodes the mature protease of SEQ ID NO:9. In some embodiments, the modified full-length protease is a serine protease that is derived from a wild-type or a variant parent serine protease *e.g.* a *Bacillus subtilis*, a *Bacillus amyloliquefaciens,* a *Bacillus pumilis* or a *Bacillus licheniformis* serine protease. Preferably, the host cell is a *Bacillus* sp. host cell *e.g.* a *Bacillus subtilis* host cell.

[008] The present invention also provides an isolated modified polynucleotide that encodes a modified full-length protease, wherein the isolated modified polynucleotide comprises a first polynucleotide that encodes the pre-pro region of the full-length protease, and that is operably linked to a second polynucleotide that encodes the mature region of the full-length protease, wherein the first polynucleotide encodes the pre-pro region of SEQ ID NO:7, which is further mutated to comprise at least one mutation that enhances the production of the protease by a host cell. In some embodiments, the at least one mutation of the first polynucleotide encodes at least one amino acid substitution at one or more positions selected from positions 2, 3, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 57, 58, 59, 61, 62, 63, 64, 66, 67, 68, 69, 70, 72, 74, 75, 76, 77, 78, 80, 82, 83, 84, 87, 88, 89, 90, 91, 93, 96, 100, and 102, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. In other embodiments, the at least one mutation encodes at least one substitution selected from X2F, N, P, and Y; X3A, M, P, and R; X6K, and M; X7E; I8W; X10A, C, G, M, and T; X11A, F, and T; X12C, P, T; X13C, G, and S; X14F; X15G, M, T, and V; X16V; X17S; X19P, and S; X20V;

X21S; X22E; X23F, Q, and W; X24G, T and V; X25A, D, and W; X26C, and H; X27A, F, H, P, T, V, and Y; X28V; X29E, I, R, S, and T; X30C; X31H, K, N, S, V, and W; X32C, F, M, N, P, S, and V; X33E, F, M, P, and S; X34D, H, P, and V; X35C, Q, and S; X36C, D, L, N, S, W, and Y; X37C, G, K, and Q; X38F, Q, S, and W; X39A, C, G, I, L, M, P, S, T, and V; X45G and S; X46S; X47E and F; X48G, I, T, W, and Y; X49A, C, E and I; X50D, and Y; X51A and H; X52A, H, I, and M; X53D, E, M, Q, and T; X54F, G, H, I, and S; X55D; X57E, N, and R; X58A, C, E, F, G, K, R, S, T, W; X59E; X61A, F, I, and R; X62A, F, G, H, N, S, T and V; X63A, C, E, F, G, N, Q, R, and T; G64D, M, Q, and S; X66E; X67G and L; X68C, D, and R; X69Y; X70E, G, K, L, M, P, S, and V; X72D and N; X74C and Y; X75G; X76V; X77E, V, and Y; X78M, Q and V; X80D, L, and N; X82C, D, P, Q, S, and T; X83G, and N; X84M; X87R; X88A, D, G, T, and V; X89V; X90D and Q; X91A; X92E and S; X93G, N, and S; X96G, N, and T; X100Q; and X102T, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. In some other embodiments, the at least one mutation encodes at least one substitution selected from R2F, N, P, and Y; S3A, M, P, and R; L6K, and M; W7E; I8W; L10A, C, G, M, and T; L11A, F, and T; F12C, P, T; A13C, G, and S; L14F; A15G, M, T, and V; L16V; I17S; T19P, and S; M20V; A21S; F22E; G23F, Q, and W; S24G, T and V; T25A, D, and W; S26C, and H; S27A, F, H, P, T, V, and Y; A28V; Q29E, I, R, S, and T; A30C; A31H, K, N, S, V, and W; G32C, F, M, N, P, S, and T; K33E, F, M, P, and S; S34D, H, P, and V; N35C, Q, and S; G36C, D, L, N, S, W, and Y; E37C, G, K, and Q; K38F, Q, S, and W; K39A, C, G, I, L, M, P, S, T, and V; K45G and S; Q46S; T47E and F; M48G, I, T, W, and Y; S49A, C, E and I; T50D, and Y; M51A and H; S52A, H, I, and M; A53D, E, M, Q, and T; A54F, G, H, I, and S; K55D; K57E, N, and R; D58A, C, E, F, G, K, R, S, T, W; V59E; S61A, F, I, and R; E62A, F, G, H, N, S, T and V; K63A, C, E, F, G, N, Q, R, and T; 64D, M, Q, and S; K66E; V67G and L; Q68C, D, and R; K69Y; Q70E, G, K, L, M, P, S, and V; K72D and N; V74C and Y; D75G; A76V; A77E, V, and Y; S78M, Q and V; T80D, L, and N; N82C, D, P, Q, S, and T; E83G, and N; K84M; K87R; E88A, D, G, T, and V; L89V; K90D and Q; K91A; D92E and S; P93G, N, and S; A96G, N, and T; E100Q; and H102T, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. The host cell is a *Bacillus* sp. host cell *e.g.* a *Bacillus subtilis* host cell. The modified full-length protease is a serine protease that is derived from a wild-type or a variant parent serine protease. In some embodiments, the wild-type or variant parent serine protease is a *Bacillus subtilis*, a *Bacillus amyloliquefaciens,* a *Bacillus pumilis* or a *Bacillus licheniformis* serine protease. In some embodiments, the second polynucleotide encodes a protease that has at least about 65% identity to the protease of SEQ ID NO:9. Preferably, the second polynucleotide encodes the mature protease of SEQ ID NO:9.

[009] The present invention also provides an isolated modified polynucleotide that encodes a modified full-length protease, wherein the isolated modified polynucleotide comprises a first polynucleotide that encodes the pre-pro region of the full-length protease, and that is operably linked to a second polynucleotide that encodes the mature region of the full-length protease, wherein the first polynucleotide encodes the pre-pro region of SEQ ID NO:7, which is further mutated to comprise at least one mutation that enhances the production of the protease by a host cell. The at least one mutation of the first polynucleotide encodes a combination of mutations that encodes a combination of

substitutions selected from X49A-X24T, X49A-X72D, X49A-X78M, X49A-X78V, X49A-X93S, X49C-X24T, X49C-X72D, X49C-X78M, X49C-X78V, X49C-X91A, X49C-X93S, X91A-x24T, X91A-X49A, X91A-X52H, X91A-X72D, X91A-X78M, X91A-X78V, X93S-X24T, X93S-X49C, X93S-X52H, X93S-X72D, X93S-X78M, and X93S-X78V, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. In other embodiments, the at least one mutation that is a combination of mutations that encodes a combination of substitutions is selected from S49A-S24T, S49A-K72D, S49A-S78M, S49A-S78V, S49A-P93S, S49C-S24T, S49C-K72D, S49C-S78M, S49C-S78V, S49C-K91A, S49C-P93S, K91A-S24T, K91A-S49A, K91A-S52H, K91A-K72D, K91A-S78M, K91A-S78V, P93S-S24T, P93S-S49C, P93S-S52H, P93S-K72D, P93S-S78M, and P93S-S78V, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. The host cell is a *Bacillus* sp. host cell *e.g.* a *Bacillus subtilis* host cell. The modified full-length protease is a serine protease that is derived from a wild-type or a variant parent serine protease. In some embodiments, the wild-type or variant parent serine protease is a *Bacillus subtilis*, a *Bacillus amyloliquefaciens,* a *Bacillus pumilis* or a *Bacillus licheniformis* serine protease. In some embodiments, the second polynucleotide encodes a protease that has at least about 65% identity to the protease of SEQ ID NO:9. Preferably, the second polynucleotide encodes the mature protease of SEQ ID NO:9.

[0010] The present invention also provides an isolated modified polynucleotide that encodes a modified full-length protease, wherein the isolated modified polynucleotide comprises a first polynucleotide that encodes the pre-pro region of the full-length protease, and that is operably linked to a second polynucleotide that encodes the mature region of the full-length protease, wherein the first polynucleotide encodes the pre-pro region of SEQ ID NO:7, which is further mutated to comprise at least one mutation that enhances the production of the protease by a host cell. The at least one mutation of the first polynucleotide of the first polynucleotide encodes at least one deletion selected from p.X18_X19del, p.X22_23del, pX37del, pX49del, p.X47del, pX55del and p.X57del, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. In some embodiments, the at least one mutation encodes at least one deletion selected from p.I18_T19del, p.F22_G23del, p.E37del, p.T47del, p.S49del, p.K55del, and p.K57del, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. The host cell is a *Bacillus* sp. host cell *e.g.* a *Bacillus subtilis* host cell. The modified full-length protease is a serine protease that is derived from a wild-type or a variant parent serine protease. In some embodiments, the wild-type or variant parent serine protease is a *Bacillus subtilis*, a *Bacillus amyloliquefaciens,* a *Bacillus pumilis* or a *Bacillus licheniformis* serine protease. In some embodiments, the second polynucleotide encodes a protease that has at least about 65% identity to the protease of SEQ ID NO:9. Preferably, the second polynucleotide encodes the mature protease of SEQ ID NO:9.

[0011] The present invention also provides an isolated modified polynucleotide that encodes a modified full-length protease, wherein the isolated modified polynucleotide comprises a first polynucleotide that encodes the pre-pro region of the full-length protease, and that is operably linked to a second polynucleotide that encodes the mature region of the full-length protease, wherein the first polynucleotide encodes the pre-pro region of SEQ ID NO:7, which is further mutated to comprise at least one mutation that enhances the production of the protease by a host cell. The at least one mutation of the first polynucleotide of the first polynucleotide encodes at least one insertion selected from p.X2_X3insT, p.X30_X31insA, p.X19_X20insAT, p.X21_X22insS, p.X32_X33insG, p.X36_X37insG, and p.X58_X59insA, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. In some embodiments, the at least one mutation encodes at least one insertion selected from p.R2_S3insT, p.A30_A31insA, p.T19_M20insAT, p.A21_F22insS, p.G32_K33insG, p.G36_E37insG, and p.D58_V59insA, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. The host cell is a *Bacillus* sp. host cell *e.g.* a *Bacillus subtilis* host cell. The modified full-length protease is a serine protease that is derived from a wild-type or a variant parent serine protease. In some embodiments, the wild-type or variant parent serine protease is a *Bacillus subtilis*, a *Bacillus amyloliquefaciens,* a *Bacillus pumilis* or a *Bacillus licheniformis* serine protease. In some embodiments, the second polynucleotide encodes a protease that has at least about 65% identity to the protease of SEQ ID NO:9. Preferably, the second polynucleotide encodes the mature protease of SEQ ID NO:9.

[0012] The present invention also provides an isolated modified polynucleotide that encodes a modified full-length protease, wherein the isolated modified polynucleotide comprises a first polynucleotide that encodes the pre-pro region of the full-length protease, and that is operably linked to a second polynucleotide that encodes the mature region of the full-length protease, wherein the first polynucleotide encodes the pre-pro polypeptide of SEQ ID NO:7, which is further mutated to comprise at least two mutations that enhance the production of the protease by a host cell. The at least two mutations of the first polynucleotide encode at least one substitution and at least one deletion selected from X46H-p.X47del, X49A-p.X22_X23del, X49C-p.X22_X23del, X48I-p.X49del, X17W-p.X18_X19del, X78M-p.X22_X23del, X78V-p.X22_X23del, X78V-p.X57del, X91A-p.X22_X23del, X91A-X48I-pX49del, X91A-p.X57del, X93S-p.X22_X23del, and X93S-X48I-p.X49del, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. In some embodiments, the at least one substitution and at least one deletion are selected from Q46H-p.T47del, S49A-p.F22_G23del, S49C-p.F22_G23del, M48I-p.S49del, I17W-p.I18_T19del, S78M-p.F22_G23del, S78V-p.F22_G23del, K91A-p.F22_G23del, K91A-M48I-pS49del, K91A-p.K57del, P93S-p.F22_G23del, and P93S-M48I-p.S49del, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. The host cell is a *Bacillus* sp. host cell *e.g.* a *Bacillus subtilis* host cell. The modified full-length protease is a serine protease that is derived from a wild-type or a variant parent serine protease. In some embodiments, the wild-

type or variant parent serine protease is a *Bacillus subtilis*, a *Bacillus amyloliquefaciens,* a *Bacillus pumilis* or a *Bacillus licheniformis* serine protease. In some embodiments, the second polynucleotide encodes a protease that has at least about 65% identity to the protease of SEQ ID NO:9. Preferably, the second polynucleotide encodes the mature protease of SEQ ID NO:9.

[0013] The present invention also provides an isolated modified polynucleotide that encodes a modified full-length protease, wherein the isolated modified polynucleotide comprises a first polynucleotide that encodes the pre-pro region of the full-length protease, and that is operably linked to a second polynucleotide that encodes the mature region of the full-length protease, wherein the first polynucleotide encodes the pre-pro polypeptide of SEQ ID NO:7, which is further mutated to comprise at least two mutations that enhance the production of the protease by a host cell. The at least two mutations of the first polynucleotide encode at least one substitution and at least one insertion are selected from X49A-p.X2_X3insT, X49A-p32X_X33insG, X49A-p.X19_X20insAT, X49C-p.X19_X20insAT, X49C-p.X32_X33insG, X52H--p.X19_X20insAT, X72D-p.X19_X20insAT, X78M-p.X19_X20insAT, X78V-p.X19_X20insAT, X91A-p.X19_X20insAT, X91A- p.X32_X33insG, X93S-p.X19_X20insAT, and X93S- p.X32_X33insG, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. In some embodiments, the at least one substitution and at least one insertion are selected from S49A-p.R2_S3insT, S49A-p32G_K33insG, S49A-p.T19_M20insAT, S49C-p.T19_M20insAT, S49C-p.G32_K33insG, S49C-p.T19_M20insAT, S52H--p.T19_M20insAT, K72D-p.T19_M20insAT, S78M-p.T19_M20insAT, S78V-p.T19_M20insAT, K91A-p.T19_M20insAT, K91A-p.G32_K33insG, P93S- p.T19_M20insAT, and P93S- p.G32_K33insG, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. The host cell is a Bacillus sp. host cell e.g. a Bacillus subtilis host cell. The modified full-length protease is a serine protease that is derived from a wild-type or a variant parent serine protease. In some embodiments, the wild-type or variant parent serine protease is a Bacillus subtilis, a Bacillus amyloliquefaciens, a Bacillus pumilis or a Bacillus licheniformis serine protease. In some embodiments, the second polynucleotide encodes a protease that has at least about 65% identity to the protease of SEQ ID NO:9. Preferably, the second polynucleotide encodes the mature protease of SEQ ID NO:9.

[0014] The present invention also provides an isolated modified polynucleotide that encodes a modified full-length protease, wherein the isolated modified polynucleotide comprises a first polynucleotide that encodes the pre-pro region of the full-length protease, and that is operably linked to a second polynucleotide that encodes the mature region of the full-length protease, wherein the first polynucleotide encodes the pre-pro polypeptide of SEQ ID NO:7, which is further mutated to comprise at least two mutations that enhance the production of the protease by a host cell. The at least two mutations of the first polynucleotide encode at least one deletion and at least one insertion selected from p.X57del-p.X19_X20insAT, and p.X22_X23del-p.X2_X3insT, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. In some embodiments, the at least one deletion and the at least

one insertion are selected from pK57del-p.T19_M20insAT, and p.F22_G23del-p.R2_S3insT. Preferably, the first polynucleotide encodes the pre-pro polypeptide of SEQ ID NO:7, which is mutated to comprise at least two mutations that enhance the production of the protease by a host cell. The host cell is a *Bacillus* sp. host cell *e.g.* a *Bacillus subtilis* host cell. The modified full-length protease is a serine protease that is derived from a wild-type or a variant parent serine protease. In some embodiments, the wild-type or variant parent serine protease is a *Bacillus subtilis*, a *Bacillus amyloliquefaciens,* a *Bacillus pumilis* or a *Bacillus licheniformis* serine protease. In some embodiments, the second polynucleotide encodes a protease that has at least about 65% identity to the protease of SEQ ID NO:9. Preferably, the second polynucleotide encodes the mature protease of SEQ ID NO:9.

[0015] The present invention also provides an isolated modified polynucleotide that encodes a modified full-length protease, wherein the isolated modified polynucleotide comprises a first polynucleotide that encodes the pre-pro region of the full-length protease, and that is operably linked to a second polynucleotide that encodes the mature region of the full-length protease, wherein the first polynucleotide encodes the pre-pro polypeptide of SEQ ID NO:7, which is further mutated to comprise at least three mutations that enhance the production of the protease by a host cell. The at least three mutations of the first polynucleotide encode at least one deletion, one insertion and one substitution corresponding to p.X49del-p.X19_X20insAT-X48I, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. In some embodiments, the at least three mutations encoding at least one deletion, one insertion and one substitution correspond to p.S49del-p.T19_M20insAT-M48I, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. The host cell is a *Bacillus* sp. host cell *e.g.* a *Bacillus subtilis* host cell. The modified full-length protease is a serine protease that is derived from a wild-type or a variant parent serine protease. In some embodiments, the wild-type or variant parent serine protease is a *Bacillus subtilis*, a *Bacillus amyloliquefaciens,* a *Bacillus pumilis* or a *Bacillus licheniformis* serine protease. In some embodiments, the second polynucleotide encodes a protease that has at least about 65% identity to the protease of SEQ ID NO:9. Preferably, the second polynucleotide encodes the mature protease of SEQ ID NO:9.

[0016] In another embodiment, the invention provides for polypeptides encoded by any one of the modified full-length polynucleotides described above.

[0017] In another embodiment, the invention provides an expression vector that comprises any one of the isolated modified polynucleotides described above. In some embodiments, the expression vector further comprises an AprE promoter *e.g* SEQ ID NO:333 or SEQ ID NO:445.

[0018] In another embodiment, the invention provides a *Bacillus sp.* host cell *e.g.* Bacillus subtilis that comprises the expression vector of the invention, and capable of expressing any one of the modified polynucleotides provided above. Preferably, the expression vector is stably integrated into the genome of the host cell. In some embodiments, the host cell of the invention is a *Bacillus* sp. host cell. In some embodiments, the *Bacillus* sp. host cell is selected from *B. subtilis, B. licheniformis, B.*

lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. clausii, B. halodurans, B. megaterium, B. coagulans, B. circulans, B. lautus, and *B. thuringiensis*. In some embodiments, the *Bacillus* sp. host cell is a *B. subtilis* host cell.

[0019] In another embodiment, the invention provides a method for producing a mature protease in a *Bacillus sp.* host cell that comprises (a) providing the expression vector comprising an isolated modified polynucleotide that encodes a modified full-length protease, which comprises a first polynucleotide that encodes the pre-pro region of the full-length protease, and that is operably linked to a second polynucleotide that encodes the mature region of the full-length protease, wherein the first polynucleotide encodes the pre-pro polypeptide of SEQ ID NO:7, which is further mutated to comprise at least one mutation that enhances the production of the mature protease by the host cell, wherein the at least one mutation is selected from X2F, N, P, and Y; X3A, M, P, and R; X6K, and M; X7E; I8W; X10A, C, G, M, and T; X11A, F, and T; X12C, P, T; X13C, G, and S; X14F; X15G, M, T, and V; X16V; X17S; X19P, and S; X20V; X21S; X22E; X23F, Q, and W; X24G, T and V; X25A, D, and W; X26C, and H; X27A, F, H, P, T, V, and Y; X28V; X29E, I, R, S, and T; X30C; X31H, K, N, S, V, and W; X32C, F, M, N, P, S, and V; X33E, F, M, P, and S; X34D, H, P, and V; X35C, Q, and S; X36C, D, L, N, S, W, and Y; X37C, G, K, and Q; X38F, Q, S, and W; X39A, C, G, I, L, M, P, S, T, and V; X45G and S; X46S; X47E and F; X48G, I, T, W, and Y; X49A, C, E and I; X50D, and Y; X51A and H; X52A, H, I, and M; X53D, E, M, Q, and T; X54F, G, H, I, and S; X55D; X57E, N, and R; X58A, C, E, F, G, K, R, S, T, W; X59E; X61A, F, I, and R; X62A, F, G, H, N, S, T and V; X63A, C, E, F, G, N, Q, R, and T; G64D, M, Q, and S; X66E; X67G and L; X68C, D, and R; X69Y; X70E, G, K, L, M, P, S, and V; X72D and N; X74C and Y; X75G; X76V; X77E, V, and Y; X78M, Q and V; X80D, L, and N; X82C, D, P, Q, S, and T; X83G, and N; X84M; X87R; X88A, D, G, T, and V; X89V; X90D and Q; X91A; X92E and S; X93G, N, and S; X96G, N, and T; X100Q; X102T; X49A-X24T, X49A-X72D, X49A-X78M, X49A-X78V, X49A-X93S, X49C-X24T, X49C-X72D, X49C-X78M, X49C-X78V, X49C-X91A, X49C-X93S, X91A-x24T, X91A-X49A, X91A-X52H, X91A-X72D, X91A-X78M, X91A-X78V, X93S-X24T, X93S-X49C, X93S-X52H, X93S-X72D, X93S-X78M, X93S-X78V, p.X18_X19del, p.X22_23del, pX37del, pX49del, p.X47del, pX55del, p.X57del, p.X2_X3insT, p.X30_X31insA, p.X19_X20insAT, p.X21_X22insS, p.X32_X33insG, p.X36_X37insG, p.X58_X59insA, X46H-p.X47del, X49A-p.X22_X23del, X49C-p.X22_X23del, X48I-p.X49del, X17W-p.X18_X19del, X78M-p.X22_X23del, X78V-p.X22_X23del, X78V-p.X57del, X91A-p.X22_X23del, X91A-X48I-pX49del, X91A-p.X57del, X93S-p.X22_X23del, X93S-X48I-p.X49del, X49A-p.X2_X3insT, X49A-p32X_X33insG, X49A-p.X19_X20insAT, X49C-p.X19_X20insAT, X49C-p.X32_X33insG, X52H--p.X19_X20insAT, X72D-p.X19_X20insAT, X78M-p.X19_X20insAT, X78V-p.X19_X20insAT, X91A-p.X19_X20insAT, X91A-p.X32_X33insG, X93S- p.X19_X20insAT, X93S- p.X32_X33insG, p.X57del-p.X19_X20insAT, p.X22_X23del-p.X2_X3insT, p.X49del-p.X19_X20insAT-X48I, and p.X49del-p.X19_X20insAT-X48I, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7; (b) transforming the host cell with the expression vector, and (c) culturing the transformed host cell under suitable conditions to allow for the production of the mature protease. In some embodiments, the method further comprises recovering the mature protease. In some embodiments, the protease is an serine protease. In some

embodiments, the *Bacillus* sp.host cell is a *Bacillus subtilis* host cell. In some embodiments, the modified polynucleotide encodes a full-length protease that comprises a mature region that is at least 65% identical to SEQ ID NO:9. Preferably, the second polynucleotide encodes the mature protease of SEQ ID NO:9. The host cell is a *Bacillus* sp. host cell *e.g.* a *Bacillus subtilis* host cell. The modified full-length protease is a serine protease that is derived from a wild-type or a variant parent serine protease. In some embodiments, the wild-type or variant parent serine protease is a *Bacillus subtilis*, a *Bacillus amyloliquefaciens,* a *Bacillus pumilis* or a *Bacillus licheniformis* serine protease.

[0020] In another embodiment, the invention provides a method for producing a mature protease in a *Bacillus sp.* host cell that comprises (a) providing an expression vector, which in turn comprises a first polynucleotide of SEQ ID NO:7 that is operably linked to a second polynucleotide that encodes the pro-pro region of SEQ ID NO:9, wherein the first polynucleotide is mutated to encode at least one mutation that enhances the production of the mature protease by the cell, wherein the at least one mutation is selected from R2F, N, P, and Y; S3A, M, P, and R; L6K, and M; W7E; I8W; L10A, C, G, M, and T; L11A, F, and T; F12C, P, T; A13C, G, and S; L14F; A15G, M, T, and V; L16V; I17S; T19P, and S; M20V; A21S; F22E; G23F, Q, and W; S24G, T and V; T25A, D, and W; S26C, and H; S27A, F, H, P, T, V, and Y; A28V; Q29E, I, R, S, and T; A30C; A31H, K, N, S, V, and W; G32C, F, M, N, P, S, and T; K33E, F, M, P, and S; S34D, H, P, and V; N35C, Q, and S; G36C, D, L, N, S, W, and Y; E37C, G, K, and Q; K38F, Q, S, and W; K39A, C, G, I, L, M, P, S, T, and V; K45G and S; Q46S; T47E and F; M48G, I, T, W, and Y; S49A, C, E and I; T50D, and Y; M51A and H; S52A, H, I, and M; A53D, E, M, Q, and T; A54F, G, H, I, and S; K55D; K57E, N, and R; D58A, C, E, F, G, K, R, S, T, W; V59E; S61A, F, I, and R; E62A, F, G, H, N, S, T and V; K63A, C, E, F, G, N, Q, R, and T; 64D, M, Q, and S; K66E; V67G and L; Q68C, D, and R; K69Y; Q70E, G, K, L, M, P, S, and V; K72D and N; V74C and Y; D75G; A76V; A77E, V, and Y; S78M, Q and V; T80D, L, and N; N82C, D, P, Q, S, and T; E83G, and N; K84M; K87R; E88A, D, G, T, and V; L89V; K90D and Q; K91A; D92E and S; P93G, N, and S; A96G, N, and T; E100Q; H102T, S49A-S24T, S49A-K72D, S49A-S78M, S49A-S78V, S49A-P93S, S49C-S24T, S49C-K72D, S49C-S78M, S49C-S78V, S49C-K91A, S49C-P93S, K91A-S24T, K91A-S49A, K91A-S52H, K91A-K72D, K91A-S78M, K91A-S78V, P93S-S24T, P93S-S49C, P93S-S52H, P93S-K72D, P93S-S78M, P93S-S78V, p.I18_T19del, p.F22_G23del, p.E37del, p.T47del, p.S49del, p.K55del, p.K57del, p.R2_S3insT, p.A30_A31insA, p.T19_M20insAT, p.A21_F22insS, p.G32_K33insG, p.G36_E37insG, p.D58_V59insA, Q46H-p.T47del, S49A-p.F22_G23del, S49C-p.F22_G23del, M48I-p.S49del, I17W-p.I18_T19del, S78M-p.F22_G23del, S78V-p.F22_G23del, K91A-p.F22_G23del, K91A-M48I-pS49del, K91A-p.K57del, P93S-p.F22_G23del, P93S-M48I-p.S49del, S49A-p.R2_S3insT, S49A-p32G_K33insG, S49A-p.T19_M20insAT, S49C-p.T19_M20insAT, S49C-p.G32_K33insG, S49C-p.T19_M20insAT, S52H-p.T19_M20insAT, K72D-p.T19_M20insAT, S78M-p.T19_M20insAT, S78V-p.T19_M20insAT, K91A-p.T19_M20insAT, K91A-p.G32_K33insG, P93S- p.T19_M20insAT, P93S- p.G32_K33insG, pK57del-p.T19_M20insAT, p.F22_G23del-p.R2_S3insT, and p.S49del-p.T19_M20insAT-M48I; (b) transforming the *Bacillus* sp. host cell with the expression vector; and (c) culturing the transformed host cell under suitable conditions to allow for the production of the mature protease. In some embodiments, the method further comprises recovering the mature protease. In some embodiments, the protease is a serine

protease, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. In some embodiments, the *Bacillus* sp. host cell is a *Bacillus subtilis* host cell. In some embodiments, the at least one mutation increases the production of the mature protease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] Figure 1 provides the amino acid sequence of the full-length FNA protease of SEQ ID NO:1. Amino acids 1- 107 (SEQ ID NO:7), and amino acids 108-382 (SEQ ID NO:9) correspond to the pre-pro polypeptide and the mature portion of FNA (SEQ ID NO:1), respectively.

[0022] Figure 2 shows an alignment of the amino acid sequence of the unmodified pre-pro region of FNA (SEQ ID NO:7) with that of unmodified pre-pro regions of proteases from various *Bacillus* sp.

[0023] Figure 3 shows an alignment of the amino acid sequence of the mature region of FNA (SEQ ID NO:9) with that of mature regions of proteases from various *Bacillus* sp.

[0024] Figure 4 shows a diagram illustrating the method used for creating in-frame deletions and insertions. Library quality: 33% had no insertions or deletions; 33% had insertions and 33% had deletions; there were no frame shift mutations.

[0025] Figure 5 shows a diagram of plasmid pAC-FNAare, which was used for the expression of FNA protease in *B.subtilis*. The plasmid elements are as follows: pUB110 = DNA fragment from plasmid pUB110 [McKenzie T., Hoshino T., Tanaka T., Sueoka N. (1986) The Nucleotide Sequence of pUB110: Some Salient Features in Relation to Replication and Its Regulation. *Plasmid* **15**:93-103], pBR322 = DNA fragment from plasmid pBR322 [Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2:95-113], pC194 = DNA fragment from plasmid pC194 [Horinouchi S., Weisblum B. (1982) Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J. Bacteriol 150:815-825].

[0026] Figure 6 shows a diagram of integrating vector pJH-FNA (Ferrari et al. J. Bacteriol. 154:1513-1515 [1983]) used for expression of FNA protease in B. subtilis.

[0027] Figure 7 shows a bar diagram depicting the percent relative activity of mature FNA (SEQ ID NO:9) processed from a modified full-length FNA protein having a mutated pre-pro polypeptide containing the amino acid substitution P93S, and the deletion p.F22_G23del (clone 684) relative to the production of the same mature FNA when processed from the unmodified full-length FNA precursor protein (unmodified; SEQ ID NO:1).

DESCRIPTION OF THE INVENTION

[0028] This invention provides modified polynucleotides encoding modified proteases, and methods for altering the production of proteases in microorganisms. In particular, the modified polynucleotides comprise one or more mutations that encode modified proteases having modifications of the pre-pro region that enhance the production of the active enzyme. The present invention further relates to methods for altering the production of proteases in microorganisms, such as *Bacillus* species.

[0029] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains (e.g. Singleton and Sainsbury, Dictionary of Microbiology and Molecular Biology, 2d Ed., John Wiley and Sons, NY [1994]; and Hale and Markham, The Harper Collins Dictionary of Biology, Harper Perennial, NY [1991]). Although any methods and materials similar or equivalent to those described herein find use in the practice of the present invention, the preferred methods and materials are described herein. Accordingly, the terms defined immediately below are more fully described by reference to the Specification as a whole. Also, as used herein, the singular "a", "an" and "the" includes the plural reference unless the context clearly indicates otherwise. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context they are used by those of skill in the art.

[0030] It is intended that every maximum numerical limitation given throughout this specification include every lower numerical limitation, as if such lower numerical limitations were expressly written herein. Every minimum numerical limitation given throughout this specification will include every higher numerical limitation, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

[0031] All patents, patent applications, articles and publications mentioned herein, both supra and infra, are hereby expressly incorporated herein by reference.

[0032] Furthermore, the headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole. Nonetheless, in order to facilitate understanding of the invention, a number of terms are defined below.

Definitions

[0033] As used herein, the terms "isolated" and “purified” refer to a nucleic acid or amino acid (or other component) that is removed from at least one component with which it is naturally associated.

[0034] The term “modified polynucleotide” herein refers to a polynucleotide sequence that has been altered to contain at least one mutation to encode a “modified” protein.

[0035] As used herein, the terms “protease” and “proteolytic activity” refer to a protein or peptide exhibiting the ability to hydrolyze peptides or substrates having peptide linkages. Many well known procedures exist for measuring proteolytic activity (Kalisz, "Microbial Proteinases," In: Fiechter (ed.), Advances in Biochemical Engineering/Biotechnology, [1988]). For example, proteolytic activity may be ascertained by comparative assays which analyze the produced protease's ability to hydrolyze a

commercial substrate. Exemplary substrates useful in such analysis of protease or proteolytic activity, include, but are not limited to di-methyl casein (Sigma C-9801), bovine collagen (Sigma C-9879), bovine elastin (Sigma E-1625), and bovine keratin (ICN Biomedical 902111). Colorimetric assays utilizing these substrates are well known in the art (See e.g., WO 99/34011; and U.S. Pat. No. 6,376,450, both of which are incorporated herein by reference. The AAPF assay (See e.g., Del Mar et al., Anal. Biochem., 99:316-320 [1979]) also finds use in determining the production of mature protease. This assay measures the rate at which p-nitroaniline is released as the enzyme hydrolyzes the soluble synthetic substrate, succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (sAAPF-pNA). The rate of production of yellow color from the hydrolysis reaction is measured at 410 nm on a spectrophotometer and is proportional to the active enzyme concentration. In particular, the term "protease" herein refers to a "serine protease".

[0036] As used herein, the terms "subtilisin" and "serine protease" are used interchangeably to refer to any member of the S8 serine protease family as described in MEROPS - The Peptidase Data base (Rawlings et al., MEROPS: the peptidase database, Nucleic Acids Res, 34 Database issue, D270-272, 2006, at the website merops.sanger.ac.uk/cgi-bin/merops.cgi?id=s08;action=.). The following information was derived from MEROPS - The Peptidase Data base as of November 6, 2008 "Peptidase family S8 contains the serine endopeptidase serine protease and its homologues (Biochem J, 290:205-218, 1993). Family S8, also known as the subtilase family, is the second largest family of serine peptidases, and can be divided into two subfamilies, with subtilisin (S08.001) the type-example for subfamily S8A and kexin (S08.070) the type-example for subfamily S8B. Tripeptidyl-peptidase II (TPP-II; S08.090) was formerly considered to be the type-example of a third subfamily, but has since been determined to be misclassified. Members of family S8 have a catalytic triad in the order Asp, His and Ser in the sequence, which is a different order to that of families S1, S9 and S10. In subfamily S8A, the active site residues frequently occurs in the motifs Asp-Thr/Ser-Gly (which is similar to the sequence motif in families of aspartic endopeptidases in clan AA), His-Gly-Thr-His and Gly-Thr-Ser-Met-Ala-Xaa-Pro. In subfamily S8B, the catalytic residues frequently occur in the motifs Asp-Asp-Gly, His-Gly-Thr-Arg and Gly-Thr-Ser-Ala/Val-Ala/Ser-Pro. Most members of the S8 family are endopeptidases, and are active at neutral-mildly alkali pH. Many peptidases in the family are thermostable. Casein is often used as a protein substrate and a typical synthetic substrate is suc-AAPF. Most members of the family are nonspecific peptidases with a preference to cleave after hydrophobic residues. However, members of subfamily S8B, such as kexin (S08.070) and furin (S08.071), cleave after dibasic amino acids. Most members of the S8 family are inhibited by general serine peptidase inhibitors such as DFP and PMSF. Because many members of the family bind calcium for stability, inhibition can be seen with EDTA and EGTA, which are often thought to be specific inhibitors of metallopeptidases. Protein inhibitors include turkey ovomucoid third domain (I01.003), Streptomyces subtilisin inhibitor (I16.003), and members of family I13 such as eglin C (I13.001) and barley inhibitor CI-1A (I13.005), many of which also inhibit chymotrypsin (S01.001). The subtilisin propeptide is itself inhibitory, and the homologous proteinase B inhibitor from Saccharomyces inhibits cerevisin (S08.052). The tertiary structures for several members of family S8 have now been determined. A typical S8 protein structure consists of three layers with a seven-

stranded β sheet sandwiched between two layers of helices. Subtilisin (S08.001) is the type structure for clan SB (SB). Despite the different structure, the active sites of subtilisin and chymotrypsin (S01.001) can be superimposed, which suggests the similarity is the result of convergent rather than divergent evolution.

[0037] The terms "precursor protease" and "parent protease" herein refer to an unmodified full-length protease comprising a pre-pro region and a mature region of a full-length wild-type or variant parent protease. The precursor protease can be derived from naturally-occurring *i.e.* wild-type proteases, or from variant proteases. It is the pre-pro region of the wild-type or variant precursor protease that is modified to generate a modified protease. In this context, both "modified" and "precursor" proteases are full-length proteases comprising a signal peptide, a pro region and a mature region. The polynucleotides that encode the modified sequence are referred to as "modified polynucleotides", and the polynucleotides that encode the precursor protease are referred to as "precursor polynucleotides". "Precursor polypeptides" and "precursor polynucleotides" can be interchangeably referred to as "unmodified precursor polypeptides" or "unmodified precursor polynucleotides", respectively.

[0038] "Naturally-occurring" or "wild-type" herein refer to a protease, or a polynucleotide encoding a protease having the unmodified amino acid sequence identical to that found in nature. Naturally occurring enzymes include native enzymes, those enzymes naturally expressed or found in the particular microorganism. A sequence that is wild-type or naturally-occurring refers to a sequence from which a variant is derived. The wild-type sequence may encode either a homologous or heterologous protein.

[0039] As used herein, "variant" refers to a protein which differs from its corresponding wild-type protein by the addition of one or more amino acids to either or both the C- and N-terminal end, substitution of one or more amino acids at one or a number of different sites in the amino acid sequence, deletion of one or more amino acids at either or both ends of the protein or at one or more sites in the amino acid sequence, and/or insertion of one or more amino acids at one or more sites in the amino acid sequence. A variant protein in the context of the present invention is exemplified by the *B. amyloliquifaciens* protease FNA (SEQ ID NO:9), which is a variant of the naturally-occurring protein BPN', from which it differs by a single amino acid substitution Y217L in the mature region. Variant proteases include naturally-occurring homologs. For example, variants of the mature protease of SEQ ID NO:9 include the homologs shown in Figure 3.

[0040] The terms "derived from" and "obtained from" refer to not only a protease produced or producible by a strain of the organism in question, but also a protease encoded by a DNA sequence isolated from such strain and produced in a host organism containing such DNA sequence. Additionally, the term refers to a protease which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the protease in question. To exemplify, "proteases derived from *Bacillus*" refers to those enzymes having proteolytic activity which are naturally-produced by *Bacillus*, as well as to serine proteases like those produced by *Bacillus* sources but which through the use of genetic engineering techniques are produced by non-*Bacillus* organisms transformed with a nucleic acid encoding said serine proteases.

[0041] A "modified full-length protease" or a "modified protease" are interchangeably used to refer to a full-length protease that comprises a mature region and a pre-pro region that are derived from a parent protease, wherein the pre-pro region is mutated to contain at least one mutation. In some embodiments, the pre-pro region and the mature region are derived from the same parent protease. In other embodiments, the pre-pro region and the mature region are derived from different parent proteases. The modified protease comprises a pre-pro region that is modified to contain at least one mutation, and it is encoded by a modified polynucleotide. The amino acid sequence of the modified protease is said to be "generated" from the precursor protease amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the pre-pro region of the precursor amino acid sequence. In some embodiments, one or more amino acids of the pre-pro region of the precursor protease are substituted to generate the modified full-length protease. Such modification is of the "precursor" or the "parent" DNA sequence which encodes the amino acid sequence of the "precursor" or the "parent" protease rather than manipulation of the precursor protease per se.

[0042] The term "enhances" is used herein in reference to the effect of a mutation on the production of a mature protease from a modified precursor being greater than the production of the same mature protease when processed from an unmodified precursor.

[0043] The term "full-length protein" herein refers to a primary gene product of a gene and comprising a signal peptide, a pro sequence and a mature sequence. For example, the full-length protease of SEQ ID NO:1 comprises the signal peptide (pre region) (VRSKKLWISL LFALALIFTM AFGSTSSAQA; SEQ ID NO:3, encoded for example by the pre polynucleotide of SEQ ID NO:4), the pro region (AGKSNGEKKY IVGFKQTMST MSAAKKKDVI SEKGGKVQKQ FKYVDAASAT LNEKAVKELK KDPSVAYVEE DHVAHAY; SEQ ID NO:5, encoded for example by the pre polynucleotide GCAGGGAAATCAAACGGGGAAAAGAAATATATTGTCGGGTTTAAACAGACAATGAGCACGATGA GCGCCGCTAAGAAGAAAGATGTCATTTCTGAAAAAGGCGGGAAAGTGCAAAAGCAATTCAAATAT GTAGACGCAGCTTCAGCTACATTAAACGAAAAAGCTGTAAAAGAATTGAAAAAAGACCCGAGCGT CGCTTACGTTGAAGAAGATCACGTAGCACACGCGTAC: SEQ ID NO:6), and the mature region (SEQ ID NO:9).

[0044] The term "signal sequence", "signal peptide" or "pre region" refers to any sequence of nucleotides and/or amino acids which may participate in the secretion of the mature or precursor forms of the protein. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-terminal portion of the protein gene, which participate in the effectuation of the secretion of protein. To exemplify, a pre peptide of a protease of the present invention at least includes the amino acid sequence identical to residues 1-30 of SEQ ID NO:1.

[0045] The term "pro sequence" or "pro region" is an amino acid sequence between the signal sequence and mature protease that is necessary for the secretion/production of the protease. Cleavage of the pro sequence will result in a mature active protease. To exemplify, a pro region of a protease of the present invention at least includes the amino acid sequence identical to residues 31-107 of SEQ ID NO:1.

[0046] The term "pre-pro region" or "pre-pro polypeptide" herein refer to the N-terminal region of a protease that encompasses the pre region and the pro region of the full-length protease. To exemplify, a pre-pro region is set forth in SEQ ID NO:7, and it comprises the pro region of SEQ ID NO:5 and the signal peptide (pre region) of SEQ ID NO:3).

[0047] The terms "mature form" or "mature region" refer to the final functional portion of the protein. To exemplify, a mature form of the protease of the present invention includes the amino acid sequence identical to residues 108-382 of SEQ ID NO:1. In this context, the "mature form" is "processed from" a full-length protease, wherein the processing of the full-length protease encompasses the removal of the signal peptide and the removal of the pro region.

[0048] As used herein, "homologous protein" refers to a protein or polypeptide native or naturally occurring in a cell. Similarly, a "homologous polynucleotide" refers to a polynucleotide that is native or naturally occurring in a cell.

[0049] As used herein, the term "heterologous protein" refers to a protein or polypeptide that does not naturally occur in the host cell. Similarly, a "heterologous polynucleotide" refers to a polynucleotide that does not naturally occur in the host cell. Heterologous polypeptides and/or heterologous polynucleotides include chimeric polypeptides and/or polynucleotides.

[0050] As used herein, "substituted" and "substitutions" refer to replacement(s) of an amino acid residue or nucleic acid base in a parent sequence. In some embodiments, the substitution involves the replacement of a naturally occurring residue or base. The modified proteases herein encompass the substitution of any of the nineteen naturally occurring amino acids at any one of the amino acid residues of the pre-pro region of the precursor protease. In some embodiments, two or more amino acids are substituted to generate a modified protease that comprises a combination of amino acid substitutions. In some embodiments, combinations of substitutions are denoted by the amino acid position at which the substitution is made. For example, a combination denoted by X49A-X93S means that whichever is the amino acid (X) at position 49 in a parent protein is replaced with an alanine (A), and whichever the amino acid (X) at position 93 in a parent protein is replaced with a serine (S). Amino acid positions are given as corresponding to the numbered position in the full-length parent protein.

[0051] As used herein, "deletion" refers to loss of genetic material in which part of a sequence of DNA is missing. While any number of nucleotides can be deleted, deletion of a number of nucleotides that is not evenly divisible by three will lead to a frameshift mutation, causing all of the codons occurring after the deletion to be read incorrectly during translation, producing a severely altered and potentially nonfunctional protein. A deletion can be terminal - - a deletion that occurs towards the end of a chromosome, or a deletion can be intercalary deletion - a deletion that occurs from the interior of a gene. Deletions are denoted herein by the amino acid(s) and the position(s) of the amino acid(s) that is/are deleted. For example, p.I18del denotes that isoleucine (I) at position 18 is deleted; and p.I18_T19del denotes that both amino acids isoleucine (I) and threonine (T) at positions 18 and 19, respectively, are deleted.

[0052] Deletions of one or more amino acids can be made alone or in combination with one or more substitutions and/or insertions.

[0053] As used herein "insertion" refers to the addition of multiples of three nucleotides acids into the DNA to encode the addition of one or more amino acids in the encoded protein. Insertions are denoted herein by the amino acid(s) and the position(s) of the amino acid(s) that is/are inserted. For example, pR2_S3insT denotes that a threonine (T) is inserted between the arginine (R) at position 2 and the serine (S) at position 3. Insertions of one or more amino acids can be made alone or in combination with one or more substitutions and/or deletions.

[0054] The term "production" with reference to a protease, encompasses the two processing steps of a full-length protease including: 1. the removal of the signal peptide, which is known to occur during protein secretion; and 2. the removal of the pro region, which creates the active mature form of the enzyme and which is known to occur during the maturation process (Wang et al., Biochemistry 37:3165-3171 (1998); Power et al., Proc Natl Acad Sci USA 83:3096-3100 (1986)).

[0055] As used herein, "corresponding to," and "by correspondence" refer to a residue at the enumerated position in a protein or peptide that is equivalent to an enumerated residue in a reference protein or peptide.

[0056] The term "processed" with reference to a mature protease refers to the maturation process that a full-length protein *e.g.* a protease, undergoes to become an active mature enzyme. The term "enhanced production" herein refers to the production of a mature protease that is processed from a modified full-length protease, that occurs at a level that is greater than the level of production of the same mature protease when processed from an unmodified full-length protease.

[0057] "Activity" with respect to enzymes means "catalytic activity" and encompasses any acceptable measure of enzyme activity, such as the rate of activity, the amount of activity, or the specific activity. Catalytic activity refers to the ability to catalyze a specific chemical reaction, such as the hydrolysis of a specific chemical bond. As the skilled artisan will appreciate, the catalytic activity of an enzyme only accelerates the rate of an otherwise slow chemical reaction. Because the enzyme only acts as a catalyst, it is neither produced nor consumed by the reaction itself. The skilled artisan will also appreciate that not all polypeptides have a catalytic activity. "Specific activity" is a measure of activity of an enzyme per unit of total protein or enzyme. Thus, specific activity may be expressed by unit weight (e.g. per gram, or per milligram) or unit volume (e.g. per ml) of enzyme. Further, specific activity may include a measure of purity of the enzyme, or can provide an indication of purity, for example, where a standard of activity is known, or available for comparison. The amount of activity reflects to the amount of enzyme that is produced by the host cell that expresses the enzyme being measured.

[0058] The term "relative activity" or "ratio of production" are used herein interchangeably to refer to the ratio of the enzymatic activity of a mature protease that was processed from a modified protease to the enzymatic activity of a mature protease that was processed from an unmodified protease. The ratio of production is determined by dividing the value of the activity of the protease processed from a

modified precursor by the value of the activity of the same protease when processed from an unmodified precursor. The relative activity is the ratio of production expressed as a percentage.

[0059] As used herein, the term "expression" refers to the process by which a polypeptide is generated based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

[0060] The term "chimeric" or "fusion" when used in reference to a protein, herein refer to a protein created through the joining of two or more polynucleotides which originally coded for separate proteins. Translation of this fusion polynucleotide results in a single chimeric polynucleotide with functional properties derived from each of the original proteins. Recombinant fusion proteins are created artificially by recombinant DNA technology. A "chimeric polypeptide," or "chimera" means a protein containing sequences from more than one polypeptide. A modified protease can be chimeric in the sense that it contains a portion, region, or domain from one protease fused to one or more portions, regions, or domains from one or more other protease. By way of example, a chimeric protease might comprise a sequence for a mature protease linked to the sequence for the pre-pro peptide of another protease. The skilled artisan will appreciate that chimeric polypeptides and proteases need not consist of actual fusions of the protein sequences, but rather, polynucleotides with the corresponding encoding sequences can also be used to express chimeric polypeptides or proteases.

[0061] The term "percent (%) identity" is defined as the percentage of amino acid /nucleotide residues in a candidate sequence that are identical with the amino acid residues/ nucleotide residues of the precursor sequence (*i.e.*, the parent sequence). A % amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. Amino acid sequences may be similar, but are not "identical" where an amino acid is substituted, deleted, or inserted in the subject sequence relative to the reference sequence. For proteins, the percent sequence identity is preferably measured between sequences that are in a similar state with respect to posttranslational modification. Typically, the "mature sequence" of the subject protease, i.e. the sequence that remains after processing to remove the signal sequence and the pro region, is compared to a mature sequence of the reference protein. In other instances, a precursor sequence of a subject polypeptide sequence may be compared to the precursor of the reference sequence.

[0062] As used herein, the term "promoter" refers to a nucleic acid sequence that functions to direct transcription of a downstream gene. In some embodiments, the promoter is appropriate to the host cell in which the target gene is being expressed. The promoter, together with other transcriptional and translational regulatory nucleic acid sequences (also termed "control sequences") is necessary to express a given gene. In general, the transcriptional and translational regulatory sequences include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences.

[0063] A nucleic acid or a polypeptide is "operably linked" when it is placed into a functional relationship with another nucleic acid or polypeptide sequence, respectively. For example, a promoter

or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation; or a modified pre-pro region is operably linked to a mature region of a protease if it enables the processing of the full-length protease to produce the mature active form of the enzyme. Generally, "operably linked" means that the DNA or polypeptide sequences being linked are contiguous.

[0064] A "host cell" refers to a suitable cell that serves as a host for an expression vector comprising DNA according to the present invention. A suitable host cell may be a naturally occurring or wild-type host cell, or it may be an altered host cell. In one embodiment, the host cell is a Gram positive microorganism. In some embodiments, the term refers to cells in the genus *Bacillus*.

[0065] As used herein, "*Bacillus* sp." includes all species within the genus "*Bacillus*," as known to those of skill in the art, including but not limited to *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. pumilis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. clausii*, *B. halodurans*, *B. megaterium*, *B. coagulans*, *B. circulans*, *B. lautus*, and *B. thuringiensis*. It is recognized that the genus *Bacillus* continues to undergo taxonomical reorganization. Thus, it is intended that the genus include species that have been reclassified, including but not limited to such organisms as *B. stearothermophilus*, which is now named "*Geobacillus stearothermophilus*." The production of resistant endospores in the presence of oxygen is considered the defining feature of the genus *Bacillus*, although this characteristic also applies to the recently named *Alicyclobacillus, Amphibacillus, Aneurinibacillus, Anoxybacillus, Brevibacillus, Filobacillus, Gracilibacillus, Halobacillus, Paenibacillus, Salibacillus, Thermobacillus, Ureibacillus*, and *Virgibacillus*.

[0066] The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length. These terms include, but are not limited to, a single-, double-stranded DNA, genomic DNA, cDNA, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. Non-limiting examples of polynucleotides include genes, gene fragments, chromosomal fragments, ESTs, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

[0067] As used herein, the terms "DNA construct" and "transforming DNA" are used interchangeably to refer to DNA used to introduce sequences into a host cell or organism. The DNA construct may be generated in vitro by PCR or any other suitable technique(s) known to those in the art. In some embodiments, the DNA construct comprises a sequence of interest (e.g., a modified sequence). In some embodiments, the sequence is operably linked to additional elements such as control elements (e.g., promoters, etc.). The DNA construct may further comprise a selectable marker. In some embodiments, the DNA construct comprises sequences homologous to the host cell chromosome. In other embodiments, the DNA construct comprises non-homologous sequences. Once the DNA construct is assembled in vitro it may be used to mutagenize a region of the host cell chromosome (i.e., replace an endogenous sequence with a heterologous sequence).

[0068] As used herein, the term "expression cassette" refers to a nucleic acid construct generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a vector such as a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid sequence to be transcribed and a promoter. In some embodiments, expression vectors have the ability to incorporate and express heterologous DNA fragments in a host cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those of skill in the art. The term "expression cassette" is used interchangeably herein with "DNA construct," and their grammatical equivalents. Selection of appropriate expression vectors is within the knowledge of those of skill in the art.

[0069] As used herein, the term "heterologous DNA sequence" refers to a DNA sequence that does not naturally occur in a host cell. In some embodiments, a heterologous DNA sequence is a chimeric DNA sequence that is comprised of parts of different genes, including regulatory elements.

[0070] As used herein, the term "vector" refers to a polynucleotide construct designed to introduce nucleic acids into one or more cell types. Vectors include cloning vectors, expression vectors, shuttle vectors, and plasmids. In some embodiments, the polynucleotide construct comprises a DNA sequence encoding the full-length protease (e.g., modified protease or unmodified precursor protease). As used herein, the term "plasmid" refers to a circular double-stranded (ds) DNA construct used as a cloning vector, and which forms an extrachromosomal self-replicating genetic element in some eukaryotes or prokaryotes, or integrates into the host chromosome.

[0071] As used herein in the context of introducing a nucleic acid sequence into a cell, the term "introduced" refers to any method suitable for transferring the nucleic acid sequence into the cell. Such methods for introduction include but are not limited to protoplast fusion, transfection, transformation, conjugation, and transduction (See e.g., Ferrari et al., "Genetics," in Hardwood et al, (eds.), *Bacillus*, Plenum Publishing Corp., pages 57-72, [1989]).

[0072] As used herein, the terms "transformed" and "stably transformed" refers to a cell that has a non-native (heterologous) polynucleotide sequence integrated into its genome or as an episomal plasmid that is maintained for at least two generations.

[0073] As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

Modified Proteases

[0074] The present invention provides methods and compositions for the production of mature proteases in bacterial host cells. In particular, the invention provides compositions and methods for enhancing the production of mature serine proteases in bacterial cells. The compositions of the invention include modified polynucleotides that encode modified proteases, which have at least one

mutation in the pre-pro region, the modified serine proteases encoded by the modified polynucleotides, expression cassettes, DNA constructs, and vectors comprising the modified polynucleotides that encode the modified serine proteases, and the bacterial host cells transformed with the vectors of the invention. The methods of the invention include methods for enhancing the production of mature proteases in bacterial host cells. The produced proteases find use in the industrial production of enzymes, suitable for use in various industries, including but not limited to the cleaning, animal feed and textile processing industry.

[0075] In some embodiments, the invention provides a modified full-length polynucleotide encoding a modified full-length protease that is generated by introducing at least one mutation in the pre-pro polynucleotide derived from that encoding a wild-type or full-length variant precursor protease of animal, vegetable or microbial origin. In some embodiments, the precursor protease is of bacterial origin. In some embodiments, the precursor protease is a protease of the subtilisin type (subtilases, subtilopeptidases, EC 3.4.21.62), which comprise catalytically active amino acids, also referred to as serine proteases. In some embodiments, the precursor protease is a *Bacillus* sp. protease. Preferably, the precursor protease is a serine protease derived from *Bacillus subtilis, Bacillus amyloliquifaciens, Bacillus licheniformis* and *Bacillus pumilis*.

[0076] Examples of precursor proteases include Subtilisin BPN' (SEQ ID NO:67), which derives from Bacillus amyloliquefaciens, and is known from the work of Vasantha et al. (1984) in J. Bacteriol., Volume 159, pp. 811-819, and of J. A. Wells et al. (1983) in Nucleic Acids Research, Volume 11, pp. 7911-7925; subtilisin Carlsberg, which is described in the publications of E. L. Smith et al. (1968) in J. Biol. Chem., Volume 243, pp. 2184-2191, and of Jacobs et al. (1985) in Nucl. Acids Res., Volume 13, pp. 8913-8926, and is formed naturally by Bacillus licheniformis, Protease PB92, which is produced naturally by the alkalophilic bacterium Bacillus nov. spec. 92 ,and AprE which is produced naturally by *Bacillus subtilis*. In some embodiments, the precursor protease is FNA (SEQ ID NO:1), which is a variant of the naturally occurring BPN' from which it differs in the mature region by a single amino acid substitution at position 217 of the mature region, wherein the Tyr (Y) at position 217 of BPN' is substituted to a Leu (L) *i.e.* the 217th amino acid of the mature region of FNA is L (SEQ ID NO:9). In other embodiments, the precursor protease comprises a pre-pro region that is at least about 30% identical to that of SEQ ID NO:7 (VRSKKLWISL LFALALIFTM AFGSTSSAQA AGKSNGEKKY IVGFKQTMST MSAAKKKDVI SEKGGKVQKQ FKYVDAASAT LNEKAVKELK KDPSVAYVEE DHVAHAY; SEQ ID NO:7) operably linked to the mature region of SEQ ID NO:9 (AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNSHGT HVAGTVAALNNSIGVLGVAPSASLYAVKVLGADGSGQYSWIINGIEWAIANNMDVINMSLGGPSGSAA LKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGPELDVMA PGVSIQSTLPGNKYGALNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENTTTKLGDSFYYGKGLI NVQAAAQ; SEQ ID NO:9).

[0077] In other embodiments, the precursor protease comprises a pre-pro region that is at least about 30% identical to that of SEQ ID NO:7 operably linked a mature region that is at least about 65% of SEQ ID NO:9. In yet other embodiments, the precursor protease comprises the pre-pro region of

SEQ ID NO:7 operably linked to a mature region that is at least about 65% identical to that of SEQ ID NO:9. Examples of pre-pro regions of serine proteases that are at least about 30% identical to the pre-pro region of SEQ ID NO:7 include SEQ ID NOS:11-66 as shown in Figure 2. Examples of mature regions that are at least about 65% identical to that of SEQ ID NO:9 include SEQ ID NOS:67-122 as shown in Figure 3.

[0078] The percent identity shared by polynucleotide sequences is determined by direct comparison of the sequence information between the molecules by aligning the sequences and determining the identity by methods known in the art. An example of an algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul, *et al.,* J. Mol. Biol., 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. These initial neighborhood word hits act as starting points to find longer HSPs containing them. The word hits are expanded in both directions along each of the two sequences being compared for as far as the cumulative alignment score can be increased. Extension of the word hits is stopped when: the cumulative alignment score falls off by the quantity X from a maximum achieved value; the cumulative score goes to zero or below; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (*See,* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA 89*:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M'5, N'-4, and a comparison of both strands.

[0079] The BLAST algorithm then performs a statistical analysis of the similarity between two sequences (*See e.g.,* Karlin and Altschul, Proc. Nat'l. Acad. Sci. USA 90:5873-5787 [1993]). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a serine protease nucleic acid of this invention if the smallest sum probability in a comparison of the test nucleic acid to a serine protease nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. Where the test nucleic acid encodes a serine protease polypeptide, it is considered similar to a specified serine protease nucleic acid if the comparison results in a smallest sum probability of less than about 0.5, and more preferably less than about 0.2.

[0080] The alignments of the amino acid sequences of the pre-pro region (Figure 2) and the mature region (Figure 3) of various serine proteases to the pre-pro region and mature region of FNA were obtained using the BLAST program as follows. The pre-pro region of FNA or the mature protein region was used to search the NCBI non-redundant protein database (version February 9, 2009). The command line BLAST program (version 2.2.17) was used with default parameters except for –v 5000 and –b 5000. Only sequences that have the desired eventual percent identity were chosen. The

alignment was done using the program clustalw (version 1.83) with default parameters. The alignment was refined five times using the program MUSCLE (version 3.51) with default parameters. Only the regions corresponding to the mature region or pre-pro region of FNA are chosen in the alignment. The sequences in the alignment are ordered in deceasing order according to the percent identities to that of FNA. The percent identity was calculated as the number of identical residues aligned between the two sequences in question divided by the number of residues aligned in the alignment.

[0081] In some embodiments, the modified polynucleotides are generated from precursor polynucleotides that comprise a pre-pro polynucleotide encoding a pre-pro region that shares at least about 30%, least about 35%, least about 40%, least about 45%, least about 50%, least about 55%, least about 60%, least about 65% amino acid sequence identity, preferably at least about 70% amino acid sequence identity, more preferably at least about 75% amino acid sequence identity, still more preferably at least about 80% amino acid sequence identity, more preferably at least about 85% amino acid sequence identity, even more preferably at least about 90% amino acid sequence identity, more preferably at least about 92% amino acid sequence identity, yet more preferably at least about 95% amino acid sequence identity, more preferably at least about 97% amino acid sequence identity, still more preferably at least about 98% amino acid sequence identity, and most preferably at least about 99% amino acid sequence identity with the amino acid sequence of the pre-pro region (SEQ ID NO:7) of the precursor protease of SEQ ID NO:1 (FNA) operably linked to the polynucleotide that encodes the mature region set forth in SEQ ID NO:9. Preferably, the modified polynucleotides are generated from precursor polynucleotides that comprise a pre-pro polynucleotide that encodes the pre-pro region of SEQ ID NO:7 operably linked to the polynucleotide that encodes the mature region set forth in SEQ ID NO:9. In other embodiments, the modified polynucleotides are generated from precursor polynucleotides that encode a pre-pro region of any one of SEQ ID NOS: 11-66 operably linked to the polynucleotide that encodes the mature region set forth in SEQ ID NO:9. An example of a polynucleotide that encodes the mature protease of SEQ ID NO:9 is the polynucleotide of SEQ ID NO:10

(GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTACA
CTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGATTCTTCTCATCCTGATTTAAAG
GTAGCAGGCGGAGCCAGCATGGTTCCTTCTGAAACAAATCCTTTCCAAGACAACAACTCTCACG
GAACTCACGTTGCCGGCACAGTTGCGGCTCTTAATAACTCAATCGGTGTATTAGGCGTTGCGCC
AAGCGCATCACTTTACGCTGTAAAAGTTCTCGGTGCTGACGGTTCCGGCCAATACAGCTGGATC
ATTAACGGAATCGAGTGGGCGATCGCAAACAATATGGACGTTATTAACATGAGCCTCGGCGGAC
CTTCTGGTTCTGCTGCTTTAAAAGCGGCAGTTGATAAAGCCGTTGCATCCGGCGTCGTAGTCGTT
GCGGCAGCCGGTAACGAAGGCACTTCCGGCAGCTCAAGCACAGTGGGCTACCCTGGTAAATAC
CCTTCTGTCATTGCAGTAGGCGCTGTTGACAGCAGCAACCAAAGAGCATCTTTCTCAAGCGTAG
GACCTGAGCTTGATGTCATGGCACCTGGCGTATCTATCCAAAGCACGCTTCCTGGAAACAAATAC
GGCGCGTTGAACGGTACATCAATGGCATCTCCGCACGTTGCCGGAGCGGCTGCTTTGATTCTTT
CTAAGCACCCGAACTGGACAAACACTCAAGTCCGCAGCAGTTTAGAAAACACCACTACAAAACTT
GGTGATTCTTTCTACTATGGAAAAGGGCTGATCAACGTACAGGCGGCAGCTCAGTAA; SEQ ID NO:10).

[0082] As described above, the pre-pro region polynucleotides are further modified to introduce at least one mutation in the pre-pro region of the encoded polypeptide to enhance the level of production of the mature form of the protease when compared to the level of production of the same mature protease when processed from an unmodified polynucleotide. The modified pre-pro polynucleotides are operably linked to a mature polynucleotide to encode the modified proteases of the invention.

[0083] In some embodiments, the modified polynucleotides are generated from precursor polynucleotides that comprise a pre-pro polynucleotide encoding a pre-pro region that shares at least about 30%, least about 35%, least about 40%, least about 45%, least about 50%, least about 55%, least about 60%, least about 65% amino acid sequence identity, preferably at least about 70% amino acid sequence identity, more preferably at least about 75% amino acid sequence identity, still more preferably at least about 80% amino acid sequence identity, more preferably at least about 85% amino acid sequence identity, even more preferably at least about 90% amino acid sequence identity, more preferably at least about 92% amino acid sequence identity, yet more preferably at least about 95% amino acid sequence identity, more preferably at least about 97% amino acid sequence identity, still more preferably at least about 98% amino acid sequence identity, and most preferably at least about 99% amino acid sequence identity with the amino acid sequence of the pre-pro region (SEQ ID NO:7) of the precursor protease of SEQ ID NO:1 operably linked to the polynucleotide that encodes a mature region of a protease that shares at least about 65% amino acid sequence identity, preferably at least about 70% amino acid sequence identity, more preferably at least about 75% amino acid sequence identity, still more preferably at least about 80% amino acid sequence identity, more preferably at least about 85% amino acid sequence identity, even more preferably at least about 90% amino acid sequence identity, more preferably at least about 92% amino acid sequence identity, yet more preferably at least about 95% amino acid sequence identity, more preferably at least about 97% amino acid sequence identity, still more preferably at least about 98% amino acid sequence identity, and most preferably at least about 99% amino acid sequence identity with the amino acid sequence of the mature region (SEQ ID NO:9) of the precursor protease of SEQ ID NO:1.

[0084] In some embodiments, the modified polynucleotides are generated from a precursor polynucleotide that encodes the pro-pro region (SEQ ID NO:7) of the protease of SEQ ID NO:1 operably linked to the mature region of a protease that shares at least about 65% amino acid sequence identity, preferably at least about 70% amino acid sequence identity, more preferably at least about 75% amino acid sequence identity, still more preferably at least about 80% amino acid sequence identity, more preferably at least about 85% amino acid sequence identity, even more preferably at least about 90% amino acid sequence identity, more preferably at least about 92% amino acid sequence identity, yet more preferably at least about 95% amino acid sequence identity, more preferably at least about 97% amino acid sequence identity, still more preferably at least about 98% amino acid sequence identity, and most preferably at least about 99% amino acid sequence identity with the amino acid sequence of the mature form (SEQ ID NO:9) of the precursor protease of SEQ ID NO:1.

[0085] In yet other embodiments, the modified polynucleotides are generated from a precursor polynucleotide that encodes the pro-pro region (SEQ ID NO:7) of the protease of SEQ ID NO:1 operably linked to the mature region (SEQ ID NO:9) of the protease of SEQ ID NO:1, *i.e.* the precursor polynucleotide encodes the protease of SEQ ID NO:1. As described above, the pre-pro region polynucleotides are modified to introduce at least one mutation that enhances the level of production of the mature form of the protease when compared to the level of production of the same mature protease when processed from an unmodified polynucleotide.

[0086] The precursor polynucleotides are mutated to generate the modified polynucleotides of the invention. In some embodiments, the portion of a precursor polynucleotide sequence encoding a pre-pro region is mutated to encode at least one mutation at least at one amino acid position selected from positions 1-107, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. Thus, in some embodiments, the modified full-length polynucleotides of the invention comprise at least one mutation at least at one amino acid position selected from positions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, and 107 wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0087] In other embodiments, the modified full-length polynucleotide s comprise at least one mutation at amino acid positions 2, 3, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 57, 58, 59, 61, 62, 63, 64, 66, 67, 68, 69, 70, 72, 74, 75, 76, 77, 78, 80, 82, 83, 84, 87, 88, 89, 90, 91, 93, 96, 100, and 102, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0088] In some embodiments, the at least one mutation is a substitution chosen from the following substitutions: X2F, N, P, and Y; X3A, M, P, and R; X6K, and M; X7E; I8W; X10A, C, G, M, and T; X11A, F, and T; X12C, P, T; X13C, G, and S; X14F; X15G, M, T, and V; X16V; X17S; X19P, and S; X20V; X21S; X22E; X23F, Q, and W; X24G, T and V; X25A, D, and W; X26C, and H; X27A, F, H, P, T, V, and Y; X28V; X29E, I, R, S, and T; X30C; X31H, K, N, S, V, and W; X32C, F, M, N, P, S, and V; X33E, F, M, P, and S; X34D, H, P, and V; X35C, Q, and S; X36C, D, L, N, S, W, and Y; X37C, G, K, and Q; X38F, Q, S, and W; X39A, C, G, I, L, M, P, S, T, and V; X45G and S; X46S; X47E and F; X48G, I, T, W, and Y; X49A, C, E and I; X50D, and Y; X51A and H; X52A, H, I, and M; X53D, E, M, Q, and T; X54F, G, H, I, and S; X55D; X57E, N, and R; X58A, C, E, F, G, K, R, S, T, W; X59E; X61A, F, I, and R; X62A, F, G, H, N, S, T and V; X63A, C, E, F, G, N, Q, R, and T; G64D, M, Q, and S; X66E; X67G and L; X68C, D, and R; X69Y; X70E, G, K, L, M, P, S, and V; X72D and N; X74C and Y; X75G; X76V; X77E, V, and Y; X78M, Q and V; X80D, L, and N; X82C, D, P, Q, S, and T; X83G, and N; X84M; X87R; X88A, D, G, T, and V; X89V; X90D and Q; X91A; X92E and S; X93G, N, and S; X96G,

N, and T; X100Q; and X102T, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7. In other embodiments, the at least one mutation is a combination of substitutions chosen from X49A-X24T, X49A-X72D, X49A-X78M, X49A-X78V, X49A-X93S, X49C-X24T, X49C-X72D, X49C-X78M, X49C-X78V, X49C-X91A, X49C-X93S, X91A-x24T, X91A-X49A, X91A-X52H, X91A-X72D, X91A-X78M, X91A-X78V, X93S-X24T, X93S-X49C, X93S-X52H, X93S-X72D, X93S-X78M, and X93S-X78V, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0089] In some embodiments, the at least one mutation encodes at least one deletion selected from p.X18_X19del, p.X22_23del, pX37del, pX49del, p.X47del, pX55del and p.X57del, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0090] In some embodiments, the at least one mutation encodes at least one insertion selected from p.X2_X3insT, p.X30_X31insA, p.X19_X20insAT, p.X21_X22insS, p.X32_X33insG, p.X36_X37insG, and p.X58_X59insA, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0091] In some embodiments, the at least one mutation encodes at least one substitution and at least one deletion selected from X46H-p.X47del, X49A-p.X22_X23del, x49C-p.X22_X23del, X48I-p.X49del, X17W-p.X18_X19del, X78M-p.X22_X23del, X78V-p.X22_X23del, X78V-p.X57del, X91A-p.X22_X23del, X91A-X48I-pX49del, X91A-p.X57del, X93S-p.X22_X23del, and X93S-X48I-p.X49del, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0092] In some embodiments, the at least one mutation encodes at least one substitution and at least one insertion selected from X49A-p.X2_X3insT, X49A-p32X_X33insG, X49A-p.X19_X20insAT, X49C-p.X19_X20insAT, X49C-p.X32_X33insG, X52H--p.X19_X20insAT, X72D-p.X19_X20insAT, X78M-p.X19_X20insAT, X78V-p.X19_X20insAT, X91A-p.X19_X20insAT, X91A- p.X32_X33insG, X93S- p.X19_X20insAT, and X93S- p.X32_X33insG, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0093] In some embodiments, the at least one mutation encodes at least two mutations encoding at least one deletion and at least one insertion selected from p.X57del-p.X19_X20insAT, and p.X 22_X23del-p.X2_X3insT, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0094] In some embodiments, the at least one mutation encodes at least three mutations encoding at least one deletion, one insertion and one substitution corresponding to p.S49del-p.T19_M20insAT-M48I, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0095] In some embodiments, the precursor polynucleotide encodes the full-length FNA protease of SEQ ID NO:1. In some embodiments, the precursor polynucleotide that encodes the encodes the full-

length FNA protease of SEQ ID NO:1 is the polynucleotide of SEQ ID NO:2. Modified full-length polynucleotides are generated from the precursor polynucleotide of SEQ ID NO:2 by introducing at least one mutation in the pre-pro region (SEQ ID NO:4) of the precursor polynucleotide (SEQ ID NO:2). In some embodiments, the at least one mutation is at least one substitution chosen from at least one substitution selected from R2F, N, P, and Y; S3A, M, P, and R; L6K, and M; W7E; I8W; L10A, C, G, M, and T; L11A, F, and T; F12C, P, T; A13C, G, and S; L14F; A15G, M, T, and V; L16V; I17S; T19P, and S; M20V; A21S; F22E; G23F, Q, and W; S24G, T and V; T25A, D, and W; S26C, and H; S27A, F, H, P, T, V, and Y; A28V; Q29E, I, R, S, and T; A30C; A31H, K, N, S, V, and W; G32C, F, M, N, P, S, and T; K33E, F, M, P, and S; S34D, H, P, and V; N35C, Q, and S; G36C, D, L, N, S, W, and Y; E37C, G, K, and Q; K38F, Q, S, and W; K39A, C, G, I, L, M, P, S, T, and V; K45G and S; Q46S; T47E and F; M48G, I, T, W, and Y; S49A, C, E and I; T50D, and Y; M51A and H; S52A, H, I, and M; A53D, E, M, Q, and T; A54F, G, H, I, and S; K55D; K57E, N, and R; D58A, C, E, F, G, K, R, S, T, W; V59E; S61A, F, I, and R; E62A, F, G, H, N, S, T and V; K63A, C, E, F, G, N, Q, R, and T; 64D, M, Q, and S; K66E; V67G and L; Q68C, D, and R; K69Y; Q70E, G, K, L, M, P, S, and V; K72D and N; V74C and Y; D75G; A76V; A77E, V, and Y; S78M, Q and V; T80D, L, and N; N82C, D, P, Q, S, and T; E83G, and N; K84M; K87R; E88A, D, G, T, and V; L89V; K90D and Q; K91A; D92E and S; P93G, N, and S; A96G, N, and T; E100Q; and H102T, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0096] In some embodiments, the precursor FNA polynucleotide is mutated to encode a modified full-length FNA comprising in its pre-pro region least one combination of mutations encoding a combination of substitutions selected from S49A-S24T, S49A-K72D, S49A-S78M, S49A-S78V, S49A-P93S, S49C-S24T, S49C-K72D, S49C-S78M, S49C-S78V, S49C-K91A, S49C-P93S, K91A-S24T, K91A-S49A, K91A-S52H, K91A-K72D, K91A-S78M, K91A-S78V, P93S-S24T, P93S-S49C, P93S-S52H, P93S-K72D, P93S-S78M, and P93S-S78V, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0097] In some embodiments, the precursor FNA polynucleotide is mutated to encode a modified full-length FNA comprising in its pre-pro region at least one mutation encoding at least one deletion selected from p.I18_T19del, p.F22_G23del, p.E37del, p.T47del 466, p.S49del, p.K55del, and p.K57del, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0098] In some embodiments, the precursor FNA polynucleotide is mutated to encode a modified full-length FNA comprising in its pre-pro region at least one mutation encoding at least one insertion selected from p.R2_S3insT, p.A30_A31insA, p.T19_M20insAT, p.A21_F22insS, p.G32_K33insG, p.G36_E37insG, and p.D58_V59insA, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0099] In some embodiments, the precursor FNA polynucleotide is mutated to encode a modified full-length FNA comprising in its pre-pro region at least two mutations encoding at least one

substitution and at least one deletion selected from the group consisting of Q46H-p.T47del, S49A-p.F22_G23del, S49C-p.F22_G23del, M48I-p.S49del, I17W-p.I18_T19del, S78M-p.F22_G23del, S78V-p.F22_G23del, K91A-p.F22_G23del, K91A-M48I-pS49del, K91A-p.K57del, P93S-p.F22_G23del, and P93S-M48I-p.S49del, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0100] In some embodiments, the precursor FNA polynucleotide is mutated to encode a modified full-length FNA comprising in its pre-pro region at least two mutations encoding at least one substitution and at least one insertion selected from S49A-p.R2_S3insT, S49A-p32G_K33insG, S49A-p.T19_M20insAT, S49C-p.T19_M20insAT, S49C-p.G32_K33insG, S49C-p.T19_M20insAT, S52H--p.T19_M20insAT, K72D-p.T19_M20insAT, S78M-p.T19_M20insAT, S78V-p.T19_M20insAT, K91A-p.T19_M20insAT, K91A- p.G32_K33insG, P93S- p.T19_M20insAT, and P93S- p.G32_K33insG, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0101] In some embodiments, the precursor FNA polynucleotide is mutated to encode a modified full-length FNA comprising in its pre-pro region at least at least two mutations encoding a deletion and an insertion selected from pK57del-p.T19_M20insAT, and p.F22_G23del-p.R2_S3insT, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0102] In some embodiments, the precursor FNA polynucleotide is mutated to encode a modified full-length FNA comprising in its pre-pro region at least three mutations encoding at least one deletion, one insertion and one substitution corresponding to p.S49del-p.T19_M20insAT-M48I, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

[0103] The modification of the pre-pro region of the precursor proteases of the invention includes at least one substitution, at least one deletion, or at least one insertion. In some embodiments, the modification of the pre-pro region includes a combination of mutations. For example, modification of the pre-pro region includes a combination of at least one substitution and at least one deletion. In other embodiments, modification of the pre-pro region includes a combination of at least one substitution and at least one insertion. In other embodiments, modification of the pre-pro region includes a combination of at least one deletion and at least one insertion. In yet other embodiments, modification of the pre-pro region includes a combination of at least one substitution, at least one deletion, and at least one insertion.

[0104] Several methods are known in the art that are suitable for generating modified polynucleotide sequences of the present invention, including but not limited to site-saturation mutagenesis, scanning mutagenesis, insertional mutagenesis, deletion mutagenesis, random mutagenesis, site-directed mutagenesis, and directed-evolution, as well as various other recombinatorial approaches. The commonly used methods include DNA shuffling (Stemmer WP, Proc Natl Acad Sci U S A. 25;91(22):10747-51 [1994]), methods based on non-homologous recombination of genes e.g. ITCHY

(Ostermeier et al., Bioorg Med Chem. 7(10):2139-44 [1999]), SCRACHY (Lutz et al. Proc Natl Acad Sci U S A. 98(20):11248-53 [2001]), SHIPREC (Sieber et al., Nat Biotechnol. 19(5):456-60 [2001]),and NRR (Bittker et al., Nat Biotechnol. 20(10):1024-9 [2001]; Bittker et al., Proc Natl Acad Sci U S A. 101(18):7011-6 [2004]), and methods that rely on the use of oligonucleotides to insert random and targeted mutations, deletions and/or insertions (Ness et al., Nat Biotechnol. 20(12):1251-5 [2002]; Coco et al., Nat Biotechnol. 20(12):1246-50 [2002]; Zha et al., Chembiochem. 3;4(1):34-9 [2003], Glaser et al., J Immunol. 149(12):3903-13 [1992], Sondek and Shortle, Proc Natl Acad Sci U S A 89(8):3581-5 [1992], Yáñez et al., Nucleic Acids Res. 32(20):e158 [2004], Osuna et al., Nucleic Acids Res. 32(17):e136 [2004], Gaytán et al., Nucleic Acids Res. 29(3):E9 [2001], and Gaytán et al., Nucleic Acids Res. 30(16):e84 [2002]).

[0105] In some embodiments, the full-length parent polynucleotide is ligated into an appropriate expression plasmid, and the following mutagenesis method may be used to facilitate the construction of the modified protease of the present invention, although other methods may be used. The method is based on that described by Pisarchik et al. (Protein engineering, Design and Selection20:257-265 [2007]) with the added advantage that the restriction enzyme used herein cuts outside its recognition sequence, which allows digestion of practically any nucleotide sequence and precludes formation of a restriction site scar. First, as described herein, a naturally-occurring gene encoding the full-length protease is obtained and sequenced in whole or in part. Subsequently, the pre-pro sequence is scanned for one or more points at which it is desired to make a mutation (deletion, insertion, substitution, or a combination thereof) at one or more amino acids in the encoded pre-pro region. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by primer extension in accord with generally known methods. Fragments to the left and to the right of the desired point(s) of mutation are amplified by PCR and to include the Eam1104I restriction site. The left and right fragments are digested with Eam1104I to generate a plurality of fragments having complimentary three base overhangs, which are then pooled and ligated to generate a library of modified pre-pro sequences containing one or more mutations. The method is diagrammed in Figure 2. This method avoids the occurrence of frame-shift mutations. In addition, this method simplifies the mutagenesis process because all of the oligonucleotides can be synthesized so as to have the same restriction site, and no synthetic linkers are necessary to create the restriction sites as is required by some other methods.

[0106] As indicated above, in some embodiments, the present invention provides vectors comprising the aforementioned polynucleotides. In some embodiments, the vector is an expression vector in which the modified polynucleotide sequence encoding the modified protease of the invention is operably linked to additional segments required for efficient gene expression (e.g., a promoter operably linked to the gene of interest). In some embodiments, these necessary elements are supplied as the gene's own homologous promoter if it is recognized, (i.e., transcribed by the host), and a transcription terminator that is exogenous or is supplied by the endogenous terminator region of the protease gene. In some embodiments, a selection gene such as an antibiotic resistance gene that

enables continuous cultural maintenance of plasmid-infected host cells by growth in antimicrobial-containing media is also included.

[0107] In some embodiments, the expression vector is derived from plasmid or viral DNA, or in alternative embodiments, contains elements of both. Exemplary vectors include, but are not limited to pXX, pC194, pJH101, pE194, pHP13 (Harwood and Cutting (eds), Molecular Biological Methods for *Bacillus*, John Wiley & Sons, [1990], in particular, chapter 3; suitable replicating plasmids for *B. subtilis* include those listed on page 92; Perego, M. (1993) Integrational Vectors for Genetic Manipulations in *Bacillus subtilis*, p. 615-624; A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other Gram-positive bacteria: biochemistry, physiology and molecular genetics, American Society for Microbiology, Washington, D.C.).

[0108] For expression and production of protein(s) of interest *e.g.* a protease, in a cell, at least one expression vector comprising at least one copy of a polynucleotide encoding the modified protease, and preferably comprising multiple copies, is transformed into the cell under conditions suitable for expression of the protease. In some particularly embodiments, the sequences encoding the proteases (as well as other sequences included in the vector) are integrated into the genome of the host cell, while in other embodiments, the plasmids remain as autonomous extra-chromosomal elements within the cell. Thus, the present invention provides both extrachromosomal elements as well as incoming sequences that are integrated into the host cell genome.

[0109] In some embodiments, a replicating vector finds use in the construction of vectors comprising the polynucleotides described herein (*e.g.*, pAC-FNA; *See*, Figure 5). It is intended that each of the vectors described herein will find use in the present invention. In some embodiments, the construct is present on an integrating vector (*e.g.*, pJH-FNA; Figure 6), that enables the integration and optionally the amplification of the modified polynucleotide into the bacterial chromosome. Examples of sites for integration include, but are not limited to the *aprE*, the *amyE*, the *veg* or the *pps* regions. Indeed, it is contemplated that other sites known to those skilled in the art will find use in the present invention. In some embodiments, the promoter is the wild-type promoter for the selected precursor protease. In some other embodiments, the promoter is heterologous to the precursor protease, but is functional in the host cell. Specifically, examples of suitable promoters for use in bacterial host cells include but are not limited to the pSPAC, pAprE, pAmyE, pVeg, pHpaII promoters, the promoter of the *B. stearothermophilus* maltogenic amylase gene, the *B. amyloliquefaciens* (BAN) amylase gene, the *B. subtilis* alkaline protease gene, the *B. clausii* alkaline protease gene the *B. pumilus* xylosidase gene, the *B. thuringiensis* cryIIIA, and the *B. licheniformis* alpha-amylase gene. In some embodiments, the promoter has a sequence set forth in SEQ ID NO:333. In other embodiments, the promoter has a sequence set forth in SEQ ID NO:445. Additional promoters include, but are not limited to the A4 promoter, as well as phage Lambda P_R or P_L promoters, and the E. coli lac, trp or tac promoters.

[0110] Precursor and modified proteases are produced in host cells of any suitable Gram-positive microorganism, including bacteria and fungi. For example, in some embodiments, the modified protease is produced in host cells of fungal and/or bacterial origin. In some embodiments, the host cells are *Bacillus sp., Streptomyces sp., Escherichia sp. or Aspergillus sp.*. In some embodiments,

the modified proteases are produced by *Bacillus sp.* host cells. Examples of *Bacillus* sp. host cells that find use in the production of the modified proteins of the present invention include, but are not limited to *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens, B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus, B. coagulans*, *B. circulans, B. pumilus*, *B. thuringiensis, B. clausii,* and *B. megaterium*, as well as other organisms within the genus *Bacillus*. In some embodiments, *B. subtilis* host cells find use. U.S. Patents 5,264,366 and 4,760,025 (RE 34,606) describe various *Bacillus* host strains that find use in the present invention, although other suitable strains find use in the present invention.

[0111] Several industrial strains that find use in the present invention include non-recombinant (*i.e.,* wild-type) *Bacillus sp.* strains, as well as variants of naturally occurring strains and/or recombinant strains. In some embodiments, the host strain is a recombinant strain, wherein a polynucleotide encoding a polypeptide of interest has been introduced into the host. In some embodiments, the host strain is *a B. subtilis* host strain and particularly a recombinant *Bacillus subtilis* host strain. Numerous *B. subtilis* strains are known, including but not limited to 1A6 (ATCC 39085), 168 (1A01), SB19, W23, Ts85, B637, PB1753 through PB1758, PB3360, JH642, 1A243 (ATCC 39,087), ATCC 21332, ATCC 6051, MI113, DE100 (ATCC 39,094), GX4931, PBT 110, and PEP 211strain (*See e.g.,* Hoch *et al.*, Genetics, 73:215–228 [1973]) (*See also*, U.S. Patent No. 4,450,235; U.S. Patent No. 4,302,544; and EP 0134048; each of which is incorporated by reference in its entirety). The use of *B. subtilis* as an expression host well known in the art (*See e.g.*, *See*, Palva *et al.*, Gene 19:81-87 [1982]; Fahnestock and Fischer, J. Bacteriol., 165:796–804 [1986]; and Wang *et al.*, Gene 69:39–47 [1988]).

[0112] In some embodiments, the *Bacillus* host is a *Bacillus* sp. that includes a mutation or deletion in at least one of the following genes, *degU, degS, degR* and *degQ*. Preferably the mutation is in a *degU* gene, and more preferably the mutation is *degU(Hy)32*. (*See e.g.,* Msadek *et al.*, J. Bacteriol., 172:824-834 [1990]; and Olmos *et al.*, Mol. Gen. Genet., 253:562–567 [1997]). A preferred host strain is a *Bacillus subtilis* carrying a *degU32(Hy)* mutation. In some further embodiments, the *Bacillus* host comprises a mutation or deletion in *scoC4*, (*See, e.g.,* Caldwell *et al.*, J. Bacteriol., 183:7329-7340 [2001]); *spoIIE* (*See*, Arigoni *et al.*, Mol. Microbiol., 31:1407-1415 [1999]); and/or *oppA* or other genes of the *opp* operon (*See e.g.*,, Perego *et al.*, Mol. Microbiol., 5:173-185 [1991]). Indeed, it is contemplated that any mutation in the *opp* operon that causes the same phenotype as a mutation in the *oppA* gene will find use in some embodiments of the altered *Bacillus* strain of the present invention. In some embodiments, these mutations occur alone, while in other embodiments, combinations of mutations are present. In some embodiments, an altered *Bacillus* that can be used to produce the modified proteases of the invention is a *Bacillus* host strain that already includes a mutation in one or more of the above-mentioned genes. In addition, *Bacillus* sp. host cells that comprise mutation(s) and/or deletions of endogenous protease genes find use. In some embodiments, the *Bacillus* host cell comprises a deletion of the *aprE* and the *nprE* genes. In other embodiments, the *Bacillus* sp. host cell comprises a deletion of 5 protease genes (US20050202535), while in other embodiments, the *Bacillus sp.* host cell comprises a deletion of 9 protease genes (US20050202535).

[0113] Host cells are transformed with modified polynucleotides encoding the modified proteases of the present invention using any suitable method known in the art. Whether the modified polynucleotide is incorporated into a vector or is used without the presence of plasmid DNA, it is introduced into a microorganism, in some embodiments, preferably an *E. coli* cell or a competent *Bacillus* cell. Methods for introducing DNA into *Bacillus* cells involving plasmid constructs and transformation of plasmids into *E. coli* are well known. In some embodiments, the plasmids are subsequently isolated from *E. coli* and transformed into *Bacillus*. However, it is not essential to use intervening microorganisms such as *E. coli*, and in some embodiments, a DNA construct or vector is directly introduced into a *Bacillus* host.

[0114] Those of skill in the art are well aware of suitable methods for introducing polynucleotide sequences into *Bacillus* cells (*See e.g.*, Ferrari *et al.*, "Genetics," *in* Harwood *et al.* (ed.), *Bacillus*, Plenum Publishing Corp. [1989], pages 57-72; Saunders *et al.*, J. Bacteriol., 157:718-726 [1984]; Hoch *et al.*, J. Bacteriol., 93:1925 -1937 [1967]; Mann *et al.*, Current Microbiol., 13:131-135 [1986]; and Holubova, Folia Microbiol., 30:97 [1985]; Chang *et al.*, Mol. Gen. Genet., 168:11-115 [1979]; Vorobjeva *et al.*, FEMS Microbiol. Lett., 7:261-263 [1980]; Smith *et al.*, Appl. Env. Microbiol., 51:634 [1986]; Fisher *et al.*, Arch. Microbiol., 139:213-217 [1981]; and McDonald, J. Gen. Microbiol.,130:203 [1984]). Indeed, such methods as transformation, including protoplast transformation and congression, transduction, and protoplast fusion are known and suited for use in the present invention. Methods of transformation are used to introduce a DNA construct provided by the present invention into a host cell. Methods known in the art to transform *Bacillus*, include such methods as plasmid marker rescue transformation, which involves the uptake of a donor plasmid by competent cells carrying a partially homologous resident plasmid (Contente *et al.*, Plasmid 2:555-571 [1979]; Haima *et al.*, Mol. Gen. Genet., 223:185-191 [1990]; Weinrauch *et al.*, J. Bacteriol., 154:1077-1087 [1983]; and Weinrauch *et al.*, J. Bacteriol., 169:1205-1211 [1987]). In this method, the incoming donor plasmid recombines with the homologous region of the resident "helper" plasmid in a process that mimics chromosomal transformation.

[0115] In addition to commonly used methods, in some embodiments, host cells are directly transformed (*i.e.*, an intermediate cell is not used to amplify, or otherwise process, the DNA construct prior to introduction into the host cell). Introduction of the DNA construct into the host cell includes those physical and chemical methods known in the art to introduce DNA into a host cell without insertion into a plasmid or vector. Such methods include, but are not limited to calcium chloride precipitation, electroporation, naked DNA, liposomes and the like. In additional embodiments, DNA constructs are co-transformed with a plasmid, without being inserted into the plasmid. In further embodiments, a selective marker is deleted from the altered *Bacillus* strain by methods known in the art (*See*, Stahl *et al.*, J. Bacteriol., 158:411-418 [1984]; and Palmeros *et al.*, Gene 247:255 -264 [2000]).

[0116] In some embodiments, the transformed cells of the present invention are cultured in conventional nutrient media. The suitable specific culture conditions, such as temperature, pH and the like are known to those skilled in the art. In addition, some culture conditions may be found in the

scientific literature such as Hopwood (2000) Practical *Streptomyces* Genetics, John Innes Foundation, Norwich UK; Hardwood et al., (1990) Molecular Biological Methods for *Bacillus*, John Wiley and from the American Type Culture Collection (ATCC).

[0117] In some embodiments, host cells transformed with polynucleotide sequences encoding modified proteases are cultured in a suitable nutrient medium under conditions permitting the expression and production of the present protease, after which the resulting protease is recovered from the culture. The medium used to culture the cells comprises any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (*e.g.*, in catalogues of the American Type Culture Collection). In some embodiments, the protease produced by the cells is recovered from the culture medium by conventional procedures, including, but not limited to separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt (*e.g.*, ammonium sulfate), chromatographic purification (*e.g.*, ion exchange, gel filtration, affinity, etc.). Thus, any method suitable for recovering the protease(s) of the present invention finds use in the present invention. Indeed, it is not intended that the present invention be limited to any particular purification method.

[0118] The protein produced by a recombinant host cell comprising a modified protease of the present invention is secreted into the culture media. In some embodiments, other recombinant constructions join the heterologous or homologous polynucleotide sequences to nucleotide sequence encoding a protease polypeptide domain which facilitates purification of the soluble proteins (Kroll DJ et al (1993) DNA Cell Biol 12:441-53). Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (Porath J (1992) Protein Expr Purif 3:263-281), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen, San Diego CA) between the purification domain and the heterologous protein also find use to facilitate purification.

[0119] As indicated above, the invention provides for modified full-length polynucleotides that encode modified full-length proteases that are processed by a *Bacillus* host cell to produce the mature form at a level that is greater than that of the same mature protease when processed from an unmodified full-length enzyme by a *Bacillus* host cell grown under the same conditions. The level of production is determined by the level of activity of the secreted enzyme.

[0120] One measure of enhancement of production can be determined as relative activity, which is expressed as a percent of the ratio of the value of the enzymatic activity of the mature form when processed from the modified protease to the value of the enzymatic activity of the mature form when processed from the unmodified precursor protease. A relative activity equal or greater than 100% indicates that the mature form a protease that is processed from a modified precursor is produced at a level that is equal or greater than the level at which the same mature protease is produced but when

processed from an unmodified precursor. Thus, in some embodiments, the relative activity of a mature protease processed from the modified protease is at least about 100%, at least about 110%, at least about 120%, at least about 130%, at least about 140%, at least about 150%, at least about 160%, at least about 170%, at least about 180%, at least about 190%, at least about 200%, at least about 225%, at least about 250%, at least about 275%, at least about 300%, at least about 325%, at least about 350%, at least about 375%, at least about 400%, at least about 425%, at least about 450%, at least about 475%, at least about 500%, at least about 525%, at least about 550%, at least about 575%, at least about 600%, at least about 625%, at least about 650%, at least about 675%, at least about 700%, at least about 725%, at least about 750%, at least about 800%, at least about 825%, at least about 850%, at least about 875%, at least about 850%, at least about 875%, at least about 900%, and up to at least about 1000% or more when compared to the corresponding production of the mature form of the protease that was processed from the unmodified precursor protease. Alternatively, the relative activity is expressed as the ratio of production which is determined by dividing the value of the activity of the protease processed from a modified precursor by the value of the activity of the same protease when processed from an unmodified precursor. Thus, in some embodiments, the ratio of production of a mature protease processed from a modified precursor is at least about 1, at least about 1.1, at least about 1.2, at least about 1.3 at least about, 1.4, at least about 1.5, at least about 1.6, at least about1.7, at least about.18, at least about1.9, at least about 2, at least about 2.25, at least about 2.5, at least about 2.75, at least about 3, at least about 3.25, at least about 3.5, at least about 3.75, at least about, at least about 4.25, at least about 4.5, at least about 4.75, at least about 5, at least about 5.25, at least about 5.5, at least about 5.75, at least about 6, at least about 6.25, at least about 6.5, at least about 6.75, at least about 7, at least about 7.25, at least about 7.5, at least about 8, at least about 8.25, at least about 8.5, at least about 8.75, at least about 9, and up to at least about 10.

[0121] There are various assays known to those of ordinary skill in the art for detecting and measuring activity of proteases. In particular, assays are available for measuring protease activity that are based on the release of acid-soluble peptides from casein or hemoglobin, measured as absorbance at 280 nm or colorimetrically using the Folin method (*See e.g.*, Bergmeyer *et al.*, "Methods of Enzymatic Analysis" vol. 5, Peptidases, Proteinases and their Inhibitors, Verlag Chemie, Weinheim [1984]). Some other assays involve the solubilization of chromogenic substrates (*See e.g.*, Ward, "Proteinases," *in* Fogarty (ed.)., Microbial Enzymes and Biotechnology, Applied Science, London, [1983], pp 251-317). Other exemplary assays include, but are not limited to succinyl-Ala-Ala-Pro-Phe-para nitroanilide assay (SAAPFpNA) and the 2,4,6-trinitrobenzene sulfonate sodium salt assay (TNBS assay). Numerous additional references known to those in the art provide suitable methods (*See e.g.*, Wells *et al.,* Nucleic Acids Res. 11:7911-7925 [1983]; Christianson *et al.*, Anal. Biochem., 223:119 -129 [1994]; and Hsia *et al.*, Anal Biochem.,242:221-227 [1999]). It is not intended that the present invention be limited to any particular assay method(s).

[0122] Other means for determining the levels of production of a mature protease in a host cell include, but are not limited to methods that use either polyclonal or monoclonal antibodies specific for

the protein. Examples include, but are not limited to enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA), fluorescent immunoassays (FIA), and fluorescent activated cell sorting (FACS). These and other assays are well known in the art (*See e.g.*, Maddox *et al* ., J. Exp. Med., 158:1211 [1983]).

[0123] All publications and patents mentioned herein are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the art and/or related fields are intended to be within the scope of the present invention.

EXPERIMENTAL

[0124] The following examples are provided in order to demonstrate and further illustrate certain embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

[0125] In the experimental disclosure which follows, the following abbreviations apply: ppm (parts per million); M (molar); mM (millimolar); µM (micromolar); nM (nanomolar); mol (moles); mmol (millimoles); µmol (micromoles); nmol (nanomoles); gm (grams); mg (milligrams); µg (micrograms); pg (picograms); L (liters); ml and mL (milliliters); µl and µL (microliters); cm (centimeters); mm (millimeters); µm (micrometers); nm (nanometers); U (units); V (volts); MW (molecular weight); sec (seconds); min(s) (minute/minutes); h(s) and hr(s) (hour/hours); °C (degrees Centigrade); QS (quantity sufficient); ND (not done); NA (not applicable); rpm (revolutions per minute); w/v (weight to volume); v/v (volume to volume); g (gravity); OD (optical density); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); suc-AAPF-pNA (succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenyl-alanyl-para-nitroanilide); FNA (variant of BPN'); BPN' (*Bacillus amyloliquefaciens* subtilisin); DMSO (dimethyl sulfoxide); cDNA (copy or complementary DNA); DNA (deoxyribonucleic acid); ssDNA (single stranded DNA); dsDNA (double stranded DNA); dNTP (deoxyribonucleotide triphosphate); DTT (1,4-dithio-DL-threitol); H2O (water); dH2O (deionized water); HCl (hydrochloric acid); $MgCl_2$ (magnesium chloride); MOPS (3-[N-morpholino]propanesulfonic acid); NaCl (sodium chloride); PAGE (polyacrylamide gel electrophoresis); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); PEG (polyethylene glycol); PCR (polymerase chain reaction); PMSF (phenylmethylsulfonyl fluoride); RNA (ribonucleic acid); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl) aminomethane); SOC (2% Bacto-Tryptone, 0.5% Bacto Yeast Extract, 10 mM NaCl, 2.5 mM KCl); Terrific Broth (TB; 12 g/l Bacto Tryptone, 24 g/l glycerol, 2.31 g/l KH_2PO_4, and 12.54 g/l K_2HPO_4); OD280 (optical density at 280 nm); OD600 (optical density at 600 nm); A405 (absorbance at 405 nm); Vmax (the maximum initial velocity of an enzyme catalyzed reaction); HEPES (N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]); Tris-HCl

(tris[Hydroxymethyl]aminomethane-hydrochloride); TCA (trichloroacetic acid); HPLC (high pressure liquid chromatography); RP-HPLC (reverse phase high pressure liquid chromatography); TLC (thin layer chromatography); EDTA (ethylenediaminetetracetic acid); EtOH (ethanol); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); TAED (N,N,N'N'-tetraacetylethylenediamine);

EXAMPLE 1

Targeted ISD (Insertion Substitution Deletion) Library Construction

[0126] The method used to create a library of modified FNA polynucleotides is outlined in Figure 2 (ISD method). Two sets of oligonucleotides that evenly covered the FNA gene sequence coding for the pre-pro region (SEQ ID NO:7) of a full-length protein of 392 amino acids (SEQ ID NO:1), in both forward and reverse direction were used to amplify the left and right segments of the portion of the FNA gene that encodes the pre-pro region of FNA. Two PCR reactions (left and right segments) contained either the 5' forward or the 3' reverse gene sequence flanking oligonucleotides each in combination with the corresponding opposite priming oligonucleotides. The left fragments were amplified using a single forward primer containing an EcoRI site (P3233, TTATTGTCTCATGAGCGGATAC; SEQ ID NO:123) and reverse primers P3301r-P3404r each containing Eam104I site (SEQ ID NOS:124-227; TABLE 1). The right fragments were amplified using a single reverse primer containing an MluI restriction site (P3237, TGTCGATAACCGCTACTTTAAC; SEQ ID NO:228) and forward primers P3301f-P3401f each containing an Eam104I restriction site (SEQ ID NOS: 229-332; TABLE 2).

TABLE 1

Sequences of reverse primers used to amplify left fragments

PRIMER NAME	PRIMER SEQUENCE	SEQ ID NO:
P3301r	AACTCTTCAVNNTCTTTACCCTCTCCTTTTAAAAAA	124
P3302r	AACTCTTCAVNNCACTCTTTACCCTCTCCTTTTAAA	125
P3303r	AACTCTTCAVNNTCTCACTCTTTACCCTCTCCTTTT	126
P3304r	AACTCTTCAVNNGCTTCTCACTCTTTACCCTCTCCT	127
P3305r	AACTCTTCAVNNTTTGCTTCTCACTCTTTACCCTCT	128
P3306r	AACTCTTCAVNNTTTTTTGCTTCTCACTCTTTACCCT	129
P3307r	AACTCTTCAVNNCAATTTTTTGCTTCTCACTCTTTA	130
P3308r	AACTCTTCAVNNCCACAATTTTTTGCTTCTCACTCT	131
P3309r	AACTCTTCAVNNGATCCACAATTTTTTGCTTCTCAC	132
P3310r	AACTCTTCAVNNACTGATCCACAATTTTTTGCTTCT	133
P3311r	AACTCTTCAVNNCAAACTGATCCACAATTTTTTGCT	134

P3312r	AACTCTTCAVNNCAGCAAACTGATCCACAATTTTTT	135
P3313r	AACTCTTCAVNNAAACAGCAAACTGATCCACAATTT	136
P3314r	AACTCTTCAVNNAGCAAACAGCAAACTGATCCACAA	137
P3315r	AACTCTTCAVNNTAAAGCAAACAGCAAACTGATCCA	138
P3316r	AACTCTTCAVNNCGCTAAAGCAAACAGCAAACTGAT	139
P3317r	AACTCTTCAVNNTAACGCTAAAGCAAACAGCAAACT	140
P3318r	AACTCTTCAVNNGATTAACGCTAAAGCAAACAGCAA	141
P3319r	AACTCTTCAVNNAAAGATTAACGCTAAAGCAAACAG	142
P3320r	AACTCTTCAVNNCGTAAAGATTAACGCTAAAGCAAA	143
P3321r	AACTCTTCAVNNCATCGTAAAGATTAACGCTAAAG	144
P3322r	AACTCTTCAVNNCGCCATCGTAAAGATTAACGCTAA	145
P3323r	AACTCTTCAVNNGAACGCCATCGTAAAGATTAAC	146
P3324r	AACTCTTCAVNNGCCGAACGCCATCGTAAAGATTAA	147
P3325r	AACTCTTCAVNNGCTGCCGAACGCCATCGTAAAGAT	148
P3326r	AACTCTTCAVNNTGTGCTGCCGAACGCCATCGTAAA	149
P3327r	AACTCTTCAVNNGGATGTGCTGCCGAACGCCATCGT	150
P3328r	AACTCTTCAVNNGCTGGATGTGCTGCCGAACGCCAT	151
P3329r	AACTCTTCAVNNCGCGCTGGATGTGCTGCCGAAC	152
P3330r	AACTCTTCAVNNCTGCGCGCTGGATGTGCTGCCGAA	153
P3331r	AACTCTTCAVNNCGCCTGCGCGCTGGATGTGCTG	154
P3332r	AACTCTTCAVNNTGCCGCCTGCGCGCTGGATGTGCT	155
P3333r	AACTCTTCAVNNCCCTGCCGCCTGCGCGCTGGATGT	156
P3334r	AACTCTTCAVNNTTTCCCTGCCGCCTGCGCGCTGGA	157
P3335r	AACTCTTCAVNNTGATTTCCCTGCCGCCTGCGCGCT	158
P3336r	AACTCTTCAVNNGTTTGATTTCCCTGCCGCCTG	159
P3337r	AACTCTTCAVNNCCCGTTTGATTTCCCTGCCGCCTG	160
P3338r	AACTCTTCAVNNTTCCCCGTTTGATTTCCCTG	161
P3339r	AACTCTTCAVNNCTTTTCCCCGTTTGATTTCCCTG	162
P3340r	AACTCTTCAVNNTTTCTTTTCCCCGTTTGATTTC	163
P3341r	AACTCTTCAVNNATATTTCTTTTCCCCGTTTGATTT	164
P3342r	AACTCTTCAVNNAATATATTTCTTTTCCCCGTTTGA	165
P3343r	AACTCTTCAVNNGACAATATATTTCTTTTCCCCGTT	166
P3344r	AACTCTTCAVNNCCCGACAATATATTTCTTTTC	167
P3345r	AACTCTTCAVNNAAACCCGACAATATATTTCTTTTC	168
P3346r	AACTCTTCAVNNTTTAAACCCGACAATATATTTCTT	169
P3347r	AACTCTTCAVNNCTGTTTAAACCCGACAATATATTT	170
P3348r	AACTCTTCAVNNTGTCTGTTTAAACCCGACAATATA	171
P3349r	AACTCTTCAVNNCATTGTCTGTTTAAACCCGACAAT	172

P3350r	AACTCTTCAVNNGCTCATTGTCTGTTTAAACCCGAC	173
P3351r	AACTCTTCAVNNCGTGCTCATTGTCTGTTTAAAC	174
P3352r	AACTCTTCAVNNCATCGTGCTCATTGTCTGTTTAAA	175
P3353r	AACTCTTCAVNNGCTCATCGTGCTCATTGTCTGTTT	176
P3354r	AACTCTTCAVNNGGCGCTCATCGTGCTCATTGTCTG	177
P3355r	AACTCTTCAVNNAGCGGCGCTCATCGTGCTCATTGT	178
P3356r	AACTCTTCAVNNCTTAGCGGCGCTCATCGTGCTCAT	179
P3357r	AACTCTTCAVNNCTTCTTAGCGGCGCTCATCGTGCT	180
P3358r	AACTCTTCAVNNTTTCTTCTTAGCGGCGCTCATCGT	181
P3359r	AACTCTTCAVNNATCTTTCTTCTTAGCGGCGCTCAT	182
P3360r	AACTCTTCAVNNGACATCTTTCTTCTTAGCGGCGCT	183
P3361r	AACTCTTCAVNNAATGACATCTTTCTTCTTAGC	184
P3362r	AACTCTTCAVNNAGAAATGACATCTTTCTTCTTAGC	185
P3363r	AACTCTTCAVNNTTCAGAAATGACATCTTTCTTCTT	186
P3364r	AACTCTTCAVNNTTTTTCAGAAATGACATCTTTCTT	187
P3365r	AACTCTTCAVNNGCCTTTTTCAGAAATGACATCTTT	188
P3366r	AACTCTTCAVNNCCCGCCTTTTTCAGAAATGACATC	189
P3367r	AACTCTTCAVNNTTTCCCGCCTTTTTCAGAAATGAC	190
P3368r	AACTCTTCAVNNCACTTTCCCGCCTTTTTCAGAAAT	191
P3369r	AACTCTTCAVNNTTGCACTTTCCCGCCTTTTTCAGA	192
P3370r	AACTCTTCAVNNCTTTTGCACTTTCCCGCCTTTTTC	193
P3371r	AACTCTTCAVNNTTGCTTTTGCACTTTCCCGCCTTT	194
P3372r	AACTCTTCAVNNGAATTGCTTTTGCACTTTCC	195
P3373r	AACTCTTCAVNNTTTGAATTGCTTTTGCACTTTC	196
P3374r	AACTCTTCAVNNATATTTGAATTGCTTTTGCACTTT	197
P3375r	AACTCTTCAVNNTACATATTTGAATTGCTTTTGCAC	198
P3376r	AACTCTTCAVNNGTCTACATATTTGAATTGCTTTTG	199
P3377r	AACTCTTCAVNNTGCGTCTACATATTTGAATTGCTT	200
P3378r	AACTCTTCAVNNAGCTGCGTCTACATATTTGAATTG	201
P3379r	AACTCTTCAVNNTGAAGCTGCGTCTACATATTTGAA	202
P3380r	AACTCTTCAVNNAGCTGAAGCTGCGTCTACATATTT	203
P3381r	AACTCTTCAVNNTGTAGCTGAAGCTGCGTCTACATA	204
P3382r	AACTCTTCAVNNTAATGTAGCTGAAGCTGCGTCTAC	205
P3383r	AACTCTTCAVNNGTTTAATGTAGCTGAAGCTGCGTC	206
P3384r	AACTCTTCAVNNTTCGTTTAATGTAGCTGAAGCTGC	207
P3385r	AACTCTTCAVNNTTTTTCGTTTAATGTAGCTGAAG	208
P3386r	AACTCTTCAVNNAGCTTTTTCGTTTAATGTAGCTGA	209
P3387r	AACTCTTCAVNNTACAGCTTTTTCGTTTAATGTAG	210

P3388r	AACTCTTCAVNNTTTTACAGCTTTTTCGTTTAATGT	211
P3389r	AACTCTTCAVNNTTCTTTTACAGCTTTTTCGTTTAA	212
P3390r	AACTCTTCAVNNCAATTCTTTTACAGCTTTTTCGTT	213
P3391r	AACTCTTCAVNNTTTCAATTCTTTTACAGCTTTTTC	214
P3392r	AACTCTTCAVNNTTTTTTCAATTCTTTTACAGCTTT	215
P3393r	AACTCTTCAVNNGTCTTTTTTCAATTCTTTTACAG	216
P3394r	AACTCTTCAVNNCGGGTCTTTTTTCAATTCTTTTAC	217
P3395r	AACTCTTCAVNNGCTCGGGTCTTTTTTCAATTCTTT	218
P3396r	AACTCTTCAVNNGACGCTCGGGTCTTTTTTCAATTC	219
P3397r	AACTCTTCAVNNAGCGACGCTCGGGTCTTTTTTCAA	220
P3398r	AACTCTTCAVNNGTAAGCGACGCTCGGGTCTTTTTT	221
P3399r	AACTCTTCAVNNAACGTAAGCGACGCTCGGGTCTTT	222
P3400r	AACTCTTCAVNNTTCAACGTAAGCGACGCTCGGGTC	223
P3401r	AACTCTTCAVNNTTCTTCAACGTAAGCGACGCTC	224
P3402r	AACTCTTCAVNNATCTTCTTCAACGTAAGCGACGCT	225
P3403r	AACTCTTCAVNNGTGATCTTCTTCAACGTAAGCGAC	226
P3404r	AACTCTTCAVNNTACGTGATCTTCTTCAACGTAAG	227

TABLE 2

Sequences of forward primers used to amplify right fragments

PRIMER NAME	PRIMER SEQUENCE	SEQ ID NO:
P3301f	AACTCTTCANNBAGAAGCAAAAAATTGTGGATCAGT	229
P3302f	AACTCTTCANNBAGCAAAAAATTGTGGATCAGTTTG	230
P3303f	AACTCTTCANNBAAAAAATTGTGGATCAGTTTGCTG	231
P3304f	AACTCTTCANNBAAATTGTGGATCAGTTTGCTGTTT	232
P3305f	AACTCTTCANNBTTGTGGATCAGTTTGCTGTTTGCT	233
P3306f	AACTCTTCANNBTGGATCAGTTTGCTGTTTGCTTTA	234
P3307f	AACTCTTCANNBATCAGTTTGCTGTTTGCTTTAG	235
P3308f	AACTCTTCANNBAGTTTGCTGTTTGCTTTAGCGTTA	236
P3309f	AACTCTTCANNBTTGCTGTTTGCTTTAGCGTTAATC	237
P3310f	AACTCTTCANNBCTGTTTGCTTTAGCGTTAATCTTT	238
P3311f	AACTCTTCANNBTTTGCTTTAGCGTTAATCTTTAC	239
P3312f	AACTCTTCANNBGCTTTAGCGTTAATCTTTACGATG	240
P3313f	AACTCTTCANNBTTAGCGTTAATCTTTACGATGG	241
P3314f	AACTCTTCANNBGCGTTAATCTTTACGATGGCGTTC	242

P3315f	AACTCTTCANNBTTAATCTTTACGATGGCGTTCG	243
P3316f	AACTCTTCANNBATCTTTACGATGGCGTTCGGCAG	244
P3317f	AACTCTTCANNBTTTACGATGGCGTTCGGCAGCACA	245
P3318f	AACTCTTCANNBACGATGGCGTTCGGCAGCACATC	246
P3319f	AACTCTTCANNBATGGCGTTCGGCAGCACATCCAG	247
P3320f	AACTCTTCANNBGCGTTCGGCAGCACATCCAGC	248
P3321f	AACTCTTCANNBTTCGGCAGCACATCCAGCGCGCAG	249
P3322f	AACTCTTCANNBGGCAGCACATCCAGCGCGCAG	250
P3323f	AACTCTTCANNBAGCACATCCAGCGCGCAGGCGGCA	251
P3324f	AACTCTTCANNBACATCCAGCGCGCAGGCGGCAG	252
P3325f	AACTCTTCANNBTCCAGCGCGCAGGCGGCAGGGAAA	253
P3326f	AACTCTTCANNBAGCGCGCAGGCGGCAGGGAAATCA	254
P3327f	AACTCTTCANNBGCGCAGGCGGCAGGGAAATCAAAC	255
P3328f	AACTCTTCANNBCAGGCGGCAGGGAAATCAAAC	256
P3329f	AACTCTTCANNBGCGGCAGGGAAATCAAACGGGGAA	257
P3330f	AACTCTTCANNBGCAGGGAAATCAAACGGGGAAAAG	258
P3331f	AACTCTTCANNBGGGAAATCAAACGGGGAAAAGAAA	259
P3332f	AACTCTTCANNBAAATCAAACGGGGAAAAGAAATAT	260
P3333f	AACTCTTCANNBTCAAACGGGGAAAAGAAATATATT	261
P3334f	AACTCTTCANNBAACGGGGAAAAGAAATATATTGTC	262
P3335f	AACTCTTCANNBGGGGAAAAGAAATATATTGTC	263
P3336f	AACTCTTCANNBGAAAAGAAATATATTGTCGGGTTT	264
P3337f	AACTCTTCANNBAAGAAATATATTGTCGGGTTTAAA	265
P3338f	AACTCTTCANNBAAATATATTGTCGGGTTTAAACAG	266
P3339f	AACTCTTCANNBTATATTGTCGGGTTTAAACAGACA	267
P3340f	AACTCTTCANNBATTGTCGGGTTTAAACAGACAATG	268
P3341f	AACTCTTCANNBGTCGGGTTTAAACAGACAATGAG	269
P3342f	AACTCTTCANNBGGGTTTAAACAGACAATGAGCAC	270
P3343f	AACTCTTCANNBTTTAAACAGACAATGAGCACGATG	271
P3344f	AACTCTTCANNBAAACAGACAATGAGCACGATGAG	272
P3345f	AACTCTTCANNBCAGACAATGAGCACGATGAG	273
P3346f	AACTCTTCANNBACAATGAGCACGATGAGCGCCGCT	274
P3347f	AACTCTTCANNBATGAGCACGATGAGCGCCGCTAAG	275
P3348f	AACTCTTCANNBAGCACGATGAGCGCCGCTAAGAAG	276
P3349f	AACTCTTCANNBACGATGAGCGCCGCTAAGAAGAAA	277
P3350f	AACTCTTCANNBATGAGCGCCGCTAAGAAGAAAGAT	278
P3351f	AACTCTTCANNBAGCGCCGCTAAGAAGAAAGATGTC	279
P3352f	AACTCTTCANNBGCCGCTAAGAAGAAAGATGTCATT	280

P3353f	AACTCTTCANNBGCTAAGAAGAAAGATGTCATTTCT	281
P3354f	AACTCTTCANNBAAGAAGAAAGATGTCATTTCTGAA	282
P3355f	AACTCTTCANNBAAGAAAGATGTCATTTCTGAAAAA	283
P3356f	AACTCTTCANNBAAAGATGTCATTTCTGAAAAAG	284
P3357f	AACTCTTCANNBGATGTCATTTCTGAAAAAGG	285
P3358f	AACTCTTCANNBGTCATTTCTGAAAAAGGCGGGAAA	286
P3359f	AACTCTTCANNBATTTCTGAAAAAGGCGGGAAAGTG	287
P3360f	AACTCTTCANNBTCTGAAAAAGGCGGGAAAGTGCAA	288
P3361f	AACTCTTCANNBGAAAAAGGCGGGAAAGTGCAAAAG	289
P3362f	AACTCTTCANNBAAAGGCGGGAAAGTGCAAAAGCAA	290
P3363f	AACTCTTCANNBGGCGGGAAAGTGCAAAAGCAATTC	291
P3364f	AACTCTTCANNBGGGAAAGTGCAAAAGCAATTCAAA	292
P3365f	AACTCTTCANNBAAAGTGCAAAAGCAATTCAAATAT	293
P3366f	AACTCTTCANNBGTGCAAAAGCAATTCAAATATGTA	294
P3367f	AACTCTTCANNBCAAAAGCAATTCAAATATGTAGAC	295
P3368f	AACTCTTCANNBAAGCAATTCAAATATGTAGACGCA	296
P3369f	AACTCTTCANNBCAATTCAAATATGTAGACGCAGCT	297
P3370f	AACTCTTCANNBTTCAAATATGTAGACGCAGCTTCA	298
P3371f	AACTCTTCANNBAAATATGTAGACGCAGCTTCAGCT	299
P3372f	AACTCTTCANNBTATGTAGACGCAGCTTCAGCTACA	300
P3373f	AACTCTTCANNBGTAGACGCAGCTTCAGCTACATTA	301
P3374f	AACTCTTCANNBGACGCAGCTTCAGCTACATTAAAC	302
P3375f	AACTCTTCANNBGCAGCTTCAGCTACATTAAACGAA	303
P3376f	AACTCTTCANNBGCTTCAGCTACATTAAACGAAAAA	304
P3377f	AACTCTTCANNBTCAGCTACATTAAACGAAAAAGCT	305
P3378f	AACTCTTCANNBGCTACATTAAACGAAAAAGCTGTA	306
P3379f	AACTCTTCANNBACATTAAACGAAAAAGCTGTAAAA	307
P3380f	AACTCTTCANNBTTAAACGAAAAAGCTGTAAAAGAA	308
P3381f	AACTCTTCANNBAACGAAAAAGCTGTAAAAGAATTG	309
P3382f	AACTCTTCANNBGAAAAAGCTGTAAAAGAATTGAAA	310
P3383f	AACTCTTCANNBAAAGCTGTAAAAGAATTGAAAAAA	311
P3384f	AACTCTTCANNBGCTGTAAAAGAATTGAAAAAAGAC	312
P3385f	AACTCTTCANNBGTAAAAGAATTGAAAAAAGACCCG	313
P3386f	AACTCTTCANNBAAAGAATTGAAAAAAGACCCGAG	314
P3387f	AACTCTTCANNBGAATTGAAAAAAGACCCGAGCGTC	315
P3388f	AACTCTTCANNBTTGAAAAAAGACCCGAGCGTCGCT	316
P3389f	AACTCTTCANNBAAAAAAGACCCGAGCGTCGCTTAC	317
P3390f	AACTCTTCANNBAAAGACCCGAGCGTCGCTTACGTT	318

P3391f	AACTCTTCANNBGACCCGAGCGTCGCTTACGTTGAA	319
P3392f	AACTCTTCANNBCCGAGCGTCGCTTACGTTGAAGAA	320
P3393f	AACTCTTCANNBAGCGTCGCTTACGTTGAAGAAGAT	321
P3394f	AACTCTTCANNBGTCGCTTACGTTGAAGAAGATCAC	322
P3395f	AACTCTTCANNBGCTTACGTTGAAGAAGATCACGTA	323
P3396f	AACTCTTCANNBTACGTTGAAGAAGATCACGTAGCA	324
P3397f	AACTCTTCANNBGTTGAAGAAGATCACGTAGCACAC	325
P3398f	AACTCTTCANNBGAAGAAGATCACGTAGCACAC	326
P3399f	AACTCTTCANNBGAAGATCACGTAGCACACGCGTAC	327
P3400f	AACTCTTCANNBGATCACGTAGCACACGCGTAC	328
P3401f	AACTCTTCANNBCACGTAGCACACGCGTACGCGCAG	329
P3402f	AACTCTTCANNBGTAGCACACGCGTACGCGCAGTC	330
P3403f	AACTCTTCANNBGCACACGCGTACGCGCAGTCCGT	331
P3404f	AACTCTTCANNBCACGCGTACGCGCAGTCCGTG	332

[0127] Each amplification reaction contained 30pmol of each oligonucleotide and 100 ng of pAC-FNa10 template. Amplifications were carried out using Vent DNA polymerase (New England Biolabs). The PCR mix (20 µl) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15s and extension at 72°C for 40 s. Following amplification, left and right fragments generated by the PCR reactions were gel-purified, mixed (200 ng of each fragment), digested with Eam104I, ligated with T4 DNA ligase and amplified by flanking primers (P3233 and P3237). The resulting fragments were digested with EcoRI and MluI, and cloned into the EcoRI/MluI sites in the pAC-FNA10 plasmid (Figure 5). pAC-FNA10 was engineered to contain an MluI restriction site between the pre-pro region and the mature region of FNA. Transcription of DNA encoding precursor and modified proteases from the pAC-FNA10 plasmid was driven by the aprE short promoter

GAATTCATCTCAAAAAAATGGGTCTACTAAAATATTATTCCATCTATTACAATAAATTCACAGAATA
GTCTTTTAAGTAAGTCTACTCTGAATTTTTTTAAAAGGAGAGGGTAAAGA (SEQ ID NO:333).

Thus, the expression cassette (1307bp) that was contained in the had the polynucleotide sequence shown below (SEQ ID NO:334)

GAATTCATCTCAAAAAAATGGGTCTACTAAAATATTATTCCATCTATTACAATAAATTCACAGAATA
GTCTTTTAAGTAAGTCTACTCTGAATTTTTTTAAAAGGAGAGGGTAAAGAGTGAGAAGCAAAAAAT
TGTGGATCAGTTTGCTGTTTGCTTTAGCGTTAATCTTTACGATGGCGTTCGGCAGCACATCCAGC
GCGCAGGCGGCAGGGAAATCAAACGGGGAAAAGAAATATATTGTCGGGTTTAAACAGACAATGA
GCACGATGAGCGCCGCTAAGAAGAAAGATGTCATTTCTGAAAAAGGCGGGAAAGTGCAAAAGCA
ATTCAAATATGTAGACGCAGCTTCAGCTACATTAAACGAAAAAGCTGTAAAAGAATTGAAAAAAGA
CCCGAGCGTCGCTTACGTTGAAGAAGATCACGTAGCACACGCGTACGCGCAGTCCGTGCCTTAC
GGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTACACTGGATCAAATGTTAAAGT
AGCGGTTATCGACAGCGGTATCGATTCTTCTCATCCTGATTTAAAGGTAGCAGGCGGAGCCAGC

ATGGTTCCTTCTGAAACAAATCCTTTCCAAGACAACAACTCTCACGGAACTCACGTTGCCGGCAC
AGTTGCGGCTCTTAATAACTCAATCGGTGTATTAGGCGTTGCGCCAAGCGCATCACTTTACGCTG
TAAAAGTTCTCGGTGCTGACGGTTCCGGCCAATACAGCTGGATCATTAACGGAATCGAGTGGGC
GATCGCAAACAATATGGACGTTATTAACATGAGCCTCGGCGGACCTTCTGGTTCTGCTGCTTTAA
AAGCGGCAGTTGATAAAGCCGTTGCATCCGGCGTCGTAGTCGTTGCGGCAGCCGGTAACGAAG
GCACTTCCGGCAGCTCAAGCACAGTGGGCTACCCTGGTAAATACCCTTCTGTCATTGCAGTAGG
CGCTGTTGACAGCAGCAACCAAAGAGCATCTTTCTCAAGCGTAGGACCTGAGCTTGATGTCATG
GCACCTGGCGTATCTATCCAAAGCACGCTTCCTGGAAACAAATACGGCGCGTTGAACGGTACAT
CAATGGCATCTCCGCACGTTGCCGGAGCGGCTGCTTTGATTCTTTCTAAGCACCCGAACTGGAC
AAACACTCAAGTCCGCAGCAGTTTAGAAAACACCACTACAAAACTTGGTGATTCTTTCTACTATGG
AAAAGGGCTGATCAACGTACAGGCGGCAGCTCAGTAAACTCGAGATAAAAAACCGGCCTTGGCC
CCGCCGGTTTTTTATTATTTTTCTTCCTCCGGATCC (SEQ ID NO:334).

[0128] The cassette contains the AprE promoter (underlined), the PRE, PRO and mature regions of FNA, and the transcription terminator.

[0129] Ligation mixtures were amplified using rolling circle amplification according to the manufacturer's recommended method (Epicentre Biotech).

[0130] One hundred and three libraries containing DNA sequences encoding FNA protease with mutated pre-pro regions were transformed into a competent *Bacillus subtilis* strain (genotype: *ΔaprE, ΔnprE, spoIIE, amyE::xylRPxylAcomK-phleo*) and recovered in 1 ml of Luria Broth (LB) at 37°C for 1 hour. The bacteria were made competent by the induction of the *comK* gene under control of a xylose inducible promoter (*See e.g.*, Hahn *et al.,* Mol Microbiol, 21:763-775, 1996). The preparations were plated on LB agar plates containing 1.6% skim milk and 5 mg/l chloramphenicol, and were incubated overnight at 37°C.

[0131] One thousand clones from each of the 103 libraries that produced the largest halos were picked, precultured by incubating the individual colonies in a 16-ml tube with 3 ml of LB containing chloramphenicol at a final concentration of 5 mg/L, and incubated 4 h at 37oC with shaking at 250rpm. One milliliter of the precultured cells was added to a 250 ml shake-flask containing 25 ml of modified FNII media (7g/L Cargill Soy Flour #4, 0.275 mM MgSO4, 220 mg/L K2HPO4, 21.32 g/L Na2HPO4 7H2O, 6.1 g/L NaH2PO4.H2O, 3.6 g/L Urea, 0.5 ml/L Mazu, 35 g/L Maltrin M150 and 23.1 g/L Glucose.H2O). Shake-flasks were incubated at 37oC with shaking at 250rpm. Aliquots of the culture (200 ul) were removed every 12 h, spinned down in the bench top centrifuge for 2 min at 8000 rpm and the supernatant was frozen at -20°C. Each isolate was screened for AAPF activity using a 96-well plate assay described below.

AAPF Protease Assay in 96-well Microtiter Plates

[0132] Clones producing the largest halos were further screened for AAPF activity using a 96-well plate assay. The chosen colonies were picked and precultured by incubating the individual colonies in a 96-well flat bottom microtiter plate (MTP) with 150 ul of LB containing chloramphenicol at a final concentration of 5 mg/L, and incubated at 37°C with shaking at 220rpm. One hundred and forty

microliters of Grant's II medium (10g/L soytone, 75 g/L glucose, 3.6 g/L urea, 83.72 g/L MOPS, 7.17 g/L tricine, 3 mM K2HPO4, 0.276 mM K2SO4, 0.528 mM MgCl2, 2.9 g/L NaCl, 1.47 mg/L Trisodium Citrate Dihydrate, 0.4 mg/L $FeSO_4$.7H2O, mg/L, 0.1 mg/L $MnSO_4$.H2O, 0.1 mg/L $ZnSO_4.H_2O$, 0.05 mg/L $CuCl_2$.2H2O, 0.1 mg/L $CoCl_2$.6H2O, 0.1 mg/L Na_2MoO_4.2H2O) was placed in each well of a fresh 96-well MTP. Then 10ul of each preculture from the first MTP was added to the corresponding well in the second MTP containing the Grant's II medium. The cultures were incubated for 40 hours in a humidified chamber at 37°C with shaking at 220rpm. Following incubation, cultures were diluted from 10 to 100 times in 100 ul of Tris dilution buffer, and the AAPF activity was measured as follows.

[0133] The AAPF activity of a sample was measured as the rate of hydrolysis of N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenyl-p-nitroanilide (suc-AAPF-pNA). The reagent solutions used were: 100 mM Tris/HCl, pH 8.6, containing 0.005% TWEEN®-80 (Tris dilution buffer and 160 mM suc-AAPF-pNA in DMSO (suc-AAPF-pNA stock solution) (Sigma: S-7388). To prepare a suc-AAPF-pNA working solution, 1 ml suc-AAPF-pNA stock solution was added to 100 ml Tris/ HCl buffer and mixed well for at least 10 seconds. The assay was performed by adding 10 µl of diluted culture to each well, immediately followed by the addition of 190 µl 1 mg/ml suc-AAPF-pNA working solution. The solutions were mixed for 5 sec., and the absorbance change in kinetic mode (20 readings in 5 minutes) was read at 410 nm in an MTP reader, at 25°C. The protease activity was expressed as AU (activity = $\Delta OD \cdot min^{-1} ml^{-1}$). Relative production was calculated as the ratio of the rate of AAPF conversion for any one experimental sample divided by the rate of AAPF conversion for the control sample (wild-type pAC-FNA10).

[0134] The results of the AAPF activity of the clones identified from the ISD Library screen and having the highest AAPF activity are given in Table 3. Clones 1001 and 515 contained two mutations: a deletion and a substitution. While the deletion was intentionally introduced into the pre-pro sequence, the substitution is likely to have resulted from mis-reading errors by the DNA polymerase.

TABLE 3

Production of mature FNA (SEQ ID NO:9) processed from modified full-length FNA relative to the production of mature FNA processed from unmodified full-length FNA comprising at least one mutation in the pre-pro region

Clone #	Mutations	Relative production (%)	Pre-pro Polypeptide Sequence	Pre-pro Nucleotide sequence
UNMODIFIED FNA	NONE	100	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:7)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:8)
340	Q46H, p.T47del	364.00±13.40	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KHMSTMSAAKKKD VISEKGGKVQKQFK YVDAASATLNEKAV KELKKDPSVAYVEE DHVAHAY (SEQ ID NO:335)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACATAT GAGCACGATGAGCGCCGCTAAGAAGAA AGATGTCATTTCTGAAAAAGGCGGGAAA GTGCAAAAGCAATTCAAATATGTAGACG CAGCTTCAGCTACATTAAACGAAAAAGC TGTAAAAGAATTGAAAAAAGACCCGAGC GTCGCTTACGTTGAAGAAGATCACGTAG CACACGCGTAC (SEQ ID NO:336)
353	S49C	393.00±27.48	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMCTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGTGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG

			EDHVAHAY (SEQ ID NO:337)	GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:338)
369	Q70G	166.10±85.80	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKGF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:339)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGGGATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:340)
371	Q70L	295.10±44.50	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKLF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:341)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGTTGTTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:342)
381	S52H	20	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMHAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:343)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGCATGCCGCTAAGAA GAAAGATGTCATTTCTGAAAAAGGCGGG AAAGTGCAAAAGCAATTCAAATATGTAG ACGCAGCTTCAGCTACATTAAACGAAAA AGCTGTAAAAGAATTGAAAAAAGACCCG

				AGCGTCGCTTACGTTGAAGAAGATCACG TAGCACACGCGTAC (SEQ ID NO:344)
390	p.K55del	154.50±30.60	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKD VISEKGGKVQKQFK YVDAASATLNEKAV KELKKDPSVAYVEE DHVAHAY (SEQ ID NO:345)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCGAAGA AAGATGTCATTTCTGAAAAAGGCGGGAA AGTGCAAAAGCAATTCAAATATGTAGAC GCAGCTTCAGCTACATTAAACGAAAAAG CTGTAAAAGAATTGAAAAAAGACCCGAG CGTCGCTTACGTTGAAGAAGATCACGTA GCACACGCGTAC (SEQ ID NO:346)
416	p.E37del	75.00	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGKKYIVGFK QTMSTMSAAKKKD VISEKGGKVQKQFK YVDAASATLNEKAV KELKKDPSVAYVEE DHVAHAY (SEQ ID NO:347)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGAAG AAATATATTGTCGGGTTTAAACAGACAAT GAGCACGATGAGCGCCGCTAAGAAGAA AGATGTCATTTCTGAAAAAGGCGGGAAA GTGCAAAAGCAATTCAAATATGTAGACG CAGCTTCAGCTACATTAAACGAAAAAGC TGTAAAAGAATTGAAAAAAGACCCGAGC GTCGCTTACGTTGAAGAAGATCACGTAG CACACGCGTAC (SEQ ID NO:348)
420	Q70M	61.00±15.3	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKMF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:349)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGATGTTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:350)
422	p.G36_E37	29.00	VRSKKLWISLLFALA	GTGAGAAGCAAAAAATTGTGGATCAGTT

	insG		LIFTMAFGSTSSAQA AGKSNGGEKKYIVG FKQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:351)	TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGG GGAAAAGAAATATATTGTCGGGTTTAAA CAGACAATGAGCACGATGAGCGCCGCT AAGAAGAAAGATGTCATTTCTGAAAAAG GCGGGAAAGTGCAAAAGCAATTCAAATA TGTAGACGCAGCTTCAGCTACATTAAAC GAAAAAGCTGTAAAGGAATTGAAAAAAG ACCCGAGCGTCGCTTACGTTGAAGAAG ATCACGTAGCACACGCGTAC (SEQ ID NO:352)
425	S61F	69.00	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVIFEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:353)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTTCGAAAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:354)
426	Q70G	62.60±13.40	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKGF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:355)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCC CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGGGGTTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:356)
429	E37G	53.00	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGGKKYIVGF	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG

			KQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:357)	CAGGCGGCAGGGAAATCAAACGGGGGT AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:358)
441	E62V	58.00	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISVKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:359)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGTCAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:360)
462	p.R2_S3ins T	134.20±68.40	VRTSKKLWISLLFAL ALIFTMAFGSTSSAQ AAGKSNGEKKYIVG FKQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:361)	GTGAGAACGAGCAAAAAATTGTGGATCA GTTTGCTGTTTGCTTTAGCGTTAATCTTT ACGATGGCGTTCGGCAGCACATCCAGC GCGCAGGCGGCAGGGAAATCAAACGGG GAAAAGAAATATATTGTCGGGTTTAAAC AGACAATGAGCACGATGAGCGCCGCTA AGAAGAAAGATGTCATTTCTGAAAAAGG CGGGAAAGTGCAAAAGCAATTCAAATAT GTAGACGCAGCTTCAGCTACATTAAACG AAAAAGCTGTAAAAGAATTGAAAAAAGA CCCGAGCGTCGCTTACGTTGAAGAAGAT CACGTAGCACACGCGTAC (SEQ ID NO:362)
464	pD58_V59i nsA	46.60±22.70	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DAVISEKGGKVQKQ	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA

			FKYVDAASATLNEK AVKELKKDPSVAYV EEDHVAHAY (SEQ ID NO:363)	CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGCCGTCATTTCTGAAAAAGG CGGGAAAGTGCAAAAGCAATTCAAATAT GTAGACGCAGCTTCAGCTACATTAAACG AAAAAGCTGTAAAAGAATTGAAAAAAGA CCCGAGCGTCGCTTACGTTGAAGAAGAT CACGTAGCACACGCGTAC (SEQ ID NO:364)
466	S78V	35.04±21.20	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAAVATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:365)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTGTCGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:366)
469	p.K55del	7.70±2.50	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKD VISEKGGKVQKQFK YVDAASATLNEKAV KELKKDPSVAYVEE DHVAHA (SEQ ID NO:367)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCGAAGA AAGATGTCATTTCTGAAAAAGGCGGGAA AGTGCAAAAGCAATTCAAATATGTAGAC GCAGCTTCAGCTACATTAAACGAAAAAG CTGTAAAAGAATTGAAAAAAGACCCGAG CGTCGCTTACGTTGAAGAAGATCACGTA GCACACGCG (SEQ ID NO:368)
470	K91A	43.61±27.77	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKADPSVAYVE	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG

			EDHVAHAY (SEQ ID NO:369)	GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAGCGGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC(SEQ ID NO:370)
472	Q70E	75.4±30.5	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKEF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:371)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGGAGTTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:372)
475	S49A	33.23±24.00	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMATMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:373)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGGCCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:374)
480	S24T	75.76±35.24	VRSKKLWISLLFALA LIFTMAFGTTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:375)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCACCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC

				GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:376)
484	S78M	90.30±74.44	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAAMATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:377)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTATGGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:378)
486	P93S	118.72±14.45	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDSSVAYVE EDHVAHAY (SEQ ID NO:379)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACTC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:380)
488	p.T19_M20 insAT	9.13±5.39	VRSKKLWISLLFALA LIFTATMAFGSTSSA QAAGKSNGEKKYIV GFKQTMSTMSAAK KKDVISEKGGKVQK QFKYVDAASATLNE KAVKELKKDPSVAY VEEDHVAHAY (SEQ ID NO:381)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG GCCACGATGGCGTTCGGCAGCACATCC AGCGCGCAGGCGGCAGGGAAATCAAAC GGGGAAAAGAAATATATTGTCGGGTTTA AACAGACAATGAGCACGATGAGCGCCG CTAAGAAGAAAGATGTCATTTCTGAAAA AGGCGGGAAAGTGCAAAAGCAATTCAAA TATGTAGACGCAGCTTCAGCTACATTAA ACGAAAAAGCTGTAAAAGAATTGAAAAA AGACCCGAGCGTCGCTTACGTTGAAGA AGATCACGTAGCACACGCGTAC (SEQ ID NO:382)

504	p.T47del	56.20±12.40	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQMSTMSAAKKKD VISEKGGKVQKQFK YVDAASATLNEKAV KELKKDPSVAYVEE DHVAHAY (SEQ ID NO:383)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGAT GAGCACGATGAGCGCCGCTAAGAAGAA AGATGTCATTTCTGAAAAAGGCGGGAAA GTGCAAAAGCAATTCAAATATGTAGACG CAGCTTCAGCTACATTAAACGAAAAAGC TGTAAAAGAATTGAAAAAAGACCCGAGC GTCGCTTACGTTGAAGAAGATCACGTAG CACACGCGTAC (SEQ ID NO:384)
506	Q70G	71.50±65.30	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKGF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:385)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGGGGTTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:386)
515	M48I,p.S49 del	229.68±29.83	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTITMSAAKKKDVI SEKGGKVQKQFKY VDAASATLNEKAVK ELKKDPSVAYVEED HVAHAY (SEQ ID NO:387)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATCACGATGAGCGCCGCTAAGAAGA AAGATGTCATTTCTGAAAAAGGCGGGAA AGTGCAAAAGCAATTCAAATATGTAGAC GCAGCTTCAGCTACATTAAACGAAAAAG CTGTAAAAGAATTGAAAAAAGACCCGAG CGTCGCTTACGTTGAAGAAGATCACGTA GCACACGCGTAC (SEQ ID NO:388)
521	S52H	69.06±33.01	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG

			KQTMSTMHAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:389)	CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGCATGCCGCTAAGAA GAAAGATGTCATTTCTGAAAAAGGCGGG AAAGTGCAAAAGCAATTCAAATATGTAG ACGCAGCTTCAGCTACATTAAACGAAAA AGCTGTAAAAGAATTGAAAAAAGACCCG AGCGTCGCTTACGTTGAAGAAGATCACG TAGCACACGCGTAC (SEQ ID NO:390)
524	p.F22_G23 del	40.00±10.88	VRSKKLWISLLFALA LIFTMASTSSAQAA GKSNGEKKYIVGFK QTMSTMSAAKKKD VISEKGGKVQKQFK YVDAASATLNEKAV KELKKDPSVAYVEE DHVAHAY (SEQ ID NO:391)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGAGCACATCCAGCGCGCAGGCG GCAGGGAAATCAAACGGGGAAAAGAAA TATATTGTCGGGTTTAAACAGACAATGA GCACGATGAGCGCCGCTAAGAAGAAAG ATGTCATTTCTGAAAAAGGCGGGAAAGT GCAAAAGCAATTCAAATATGTAGACGCA GCTTCAGCTACATTAAACGAAAAAGCTG TAAAAGAATTGAAAAAAGACCCGAGCGT CGCTTACGTTGAAGAAGATCACGTAGCA CACGCGTAC (SEQ ID NO:392)
531	S49A	91.80±25.10	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMATMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:393)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGGCCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:394)
532	p.K57del	31.30±8.60	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKD VISEKGGKVQKQFK YVDAASATLNEKAV	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA

			KELKKDPSVAYVEE DHVAHAY (SEQ ID NO:395)	AGGATGTCATTTCTGAAAAAGGCGGGAA AGTGCAAAAGCAATTCAAATATGTAGAC GCAGCTTCAGCTACATTAAACGAAAAAG CTGTAAAAGAATTGAAAAAAGACCCGAG CGTCGCTTACGTTGAAGAAGATCACGTA GCACACGCGTAC (SEQ ID NO:396)
541	p.G32_K33 insG	50.01±13.55	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGGKSNGEKKYIVG FKQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:397)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGTGGGAAATCAAACGGG GAAAAGAAATATATTGTCGGGTTTAAAC AGACAATGAGCACGATGAGCGCCGCTA AGAAGAAAGATGTCATTTCTGAAAAAGG CGGGAAAGTGCAAAAGCAATTCAAATAT GTAGACGCAGCTTCAGCTACATTAAACG AAAAAGCTGTAAAAGAATTGAAAAAAGA CCCGAGCGTCGCTTACGTTGAAGAAGAT CACGTAGCACACGCGTAC (SEQ ID NO:398)
734	K72N	89.42±67.68	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKQF DYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:399)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGCAATTCGATTATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:400)
767	p.A21_F22i nsS	41.60±17.80	VRSKKLWISLLFALA LIFTMASFGSTSSAQ AAGKSNGEKKYIVG FKQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGAGTTTCGGCAGCACATCCAGC GCGCAGGCGGCAGGGAAATCAAACGGG GAAAAGAAATATATTGTCGGGTTTAAAC AGACAATGAGCACGATGAGCGCCGCTA AGAAGAAAGATGTCATTTCTGAAAAAGG CGGGAAAGTGCAAAAGCAATTCAAATAT

			NO:401)	GTAGACGCAGCTTCAGCTACATTAAACG AAAAAGCTGTAAAAGAATTGAAAAAAGA CCCGAGCGTCGCTTACGTTGAAGAAGAT CACGTAGCACACGCGTAC (SEQ ID NO:402)
771	K57L	47.40±6.90	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKLD VISEKGGKVQKQFK YVDAASATLNEKAV KELKKDPSVAYVEE DHVAHAY (SEQ ID NO:403)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGTTGGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:404)
773	p.A30_A31insA	51.00±37.70	VRSKKLWISLLFALA LIFTMAFGSTSSAQA AAGKSNGEKKYIVG FKQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:405)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCAGCACATCCAGCGCG CAGGCGGCCGCAGGGAAATCAAACGGG GAAAAGAAATATATTGTCGGGTTTAAAC AGACAATGAGCACGATGAGCGCCGCTA AGAAGAAAGATGTCATTTCTGAAAAAGG CGGGAAAGTGCAAAAGCAATTCAAATAT GTAGACGCAGCTTCAGCTACATTAAACG AAAAAGCTGTAAAAGAATTGAAAAAAGA CCCGAGCGTCGCTTACGTTGAAGAAGAT CACGTAGCACACGCGTAC (SEQ ID NO:406)
777	S24G	129.60±72.30	VRSKKLWISLLFALA LIFTMAFGGTSSAQ AAGKSNGEKKYIVG FKQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:407)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTAATCTTTACG ATGGCGTTCGGCGGCACATCCAGCGCG CAGGCGGCAGGGAAATCAAACGGGGAA AAGAAATATATTGTCGGGTTTAAACAGA CAATGAGCACGATGAGCGCCGCTAAGA AGAAAGATGTCATTTCTGAAAAAGGCGG GAAAGTGCAAAAGCAATTCAAATATGTA GACGCAGCTTCAGCTACATTAAACGAAA

				AAGCTGTAAAAGAATTGAAAAAAGACCC GAGCGTCGCTTACGTTGAAGAAGATCAC GTAGCACACGCGTAC (SEQ ID NO:408)
1001	I17W, p.I18_T19del	1.28±0.07	VRSKKLWISLLFALA LWMAFGSTSSAQA AGKSNGEKKYIVGF KQTMSTMSAAKKK DVISEKGGKVQKQF KYVDAASATLNEKA VKELKKDPSVAYVE EDHVAHAY (SEQ ID NO:409)	GTGAGAAGCAAAAAATTGTGGATCAGTT TGCTGTTTGCTTTAGCGTTATGGATGGC GTTCGGCAGCACATCCTCTGCCCAGGC GGCAGGGAAATCAAACGGGGAAAAGAA ATATATTGTCGGGTTTAAACAGACAATG AGCACGATGAGCGCCGCTAAGAAGAAA GATGTCATTTCTGAAAAAGGCGGGAAAG TGCAAAAGCAATTCAAATATGTAGACGC AGCTTCAGCTACATTAAACGAAAAAGCT GTAAAAGAATTGAAAAAAGACCCGAGCG TCGCTTACGTTGAAGAAGATCACGTAGC ACACGCGTAC (SEQ ID NO:410)

EXAMPLE 2

Generation of mutated pre-pro polypeptides comprising a combination of mutations generated by ISD

[0135] To determine the effect of combining at least two mutations in the pre-pro FNA sequence, combinations of the mutations given in Table 3 were made as follows.

[0136] The pAC-FNA10 plasmid DNAs comprising a mutant from Table 3 was used as a template for extension PCR to add another mutation also selected from mutations described in Table 3. Two PCR reactions (left and right segments) contained either the 5' forward or the 3' reverse gene sequence flanking oligonucleotides each in combination with the corresponding oppositely priming oligonucleotides. The left fragments were amplified using a single forward primer (P3234, ACCCAACTGATCTTCAGCATC; SEQ ID NO:411) and reverse primers for the particular mutation shown in Table D. The right fragments were amplified using a single reverse primer (P3242, ACCGTCAGCACCGAGAACTT; SEQ ID NO:412) and forward primers for that particular mutation shown in Table 4. Two amplified fragments (left and right) were mixed together and amplified by the forward primer containing EcoRI site (P3201, ATAGGAATTCATCTCAAAAAAATG; SEQ ID NO:413) and reverse primer containing MluI restriction site (P3237, TGTCGATAACCGCTACTTTAAC; SEQ ID NO:414).

TABLE 4

Sequences of forward and reverse primers used to amplify the left and right fragments

Mutation introduced	Primer orientation	Primer name	Primer sequence	SEQ ID NO:
Clone 541	Forward	P3468	AGGCGGCAGGTGGGAAATCAAACGGGGA AAAGAAATA	415
Clone 541	Reverse	P3469	TTTCCCCGTTTGATTTCCCACCTGCCGCC TGCGCGCTGGA	416
Clone 462	Forward	P3408	TTCCATCTATTACAATAAATTCACAGAATA GTCTTTTAAGTAAGTCTACTCT	417
Clone 462	Reverse	P3409	CTGTGAATTTATTGTAATAGATGGAA	418
Clone 515	Forward	P3446	TTTAAACAGACAATCACGATGAGCGCCGC TAAGAA	419
Clone 515	Reverse	P3447	AGCGGCGCTCATCGTGATTGTCTGTTTAA ACCCGACAATA	420
Clone 466	Forward	P3478	TGTAGACGCAGCTGTCGCTACATTAAACG AAAAAGCTGTA	421
Clone 466	Reverse	P3479	TCGTTTAATGTAGCGACAGCTGCGTCTAC ATATTTGAATT	422
Clone 469	Forward	P3480	CGATGAGCGCCGCGAAGAAAGATGTCATT TCTGAAAAA	423
Clone 469	Reverse	P3481	GAAATGACATCTTTCTTCGCGGCGCTCAT CGTGCTCA	424
Clone 470	Forward	P3482	TGTAAAAGAATTGAAAGCGGACCCGAGCG TCGCTTACGT	425
Clone 470	Reverse	P3483	GACGCTCGGGTCCGCTTTCAATTCTTTTA CAGCTTTTTCG	426
Clone 521	Forward	P3454	AATGAGCACGATGCATGCCGCTAAGAAGA AAGATGTCA	427
Clone 521	Reverse	P3455	TTCTTCTTAGCGGCATGCATCGTGCTCATT GTCTGTTTAA	428
Clone 524	Forward	P3458	AATCTTTACGATGGCGAGCACATCCAGCG CGCAGG	429
Clone 524	Reverse	P3459	CGCGCTGGATGTGCTCGCCATCGTAAAGA TTAACGCT	430
Clone 475	Forward	P3484	GGTTTAAACAGACAATGGCCACGATGAGC GCCGCTAAGA	431
Clone 475	Reverse	P3485	GCGGCGCTCATCGTGGCCATTGTCTGTTT AAACCCGACAA	432
Clone 480	Forward	P3486	ATGGCGTTCGGCACCACATCCAGCGCGC AGGCGGCA	433
Clone 480	Reverse	P3487	CTGCGCGCTGGATGTGGTGCCGAACGCC ATCGTAAAGA	434
Clone 448	Forward	P3488	GAGAAGCAAAAAATTATGGATCAGTTTGCT GTTTGCTTT	435
Clone 448	Reverse	P3489	CAGCAAACTGATCCATAATTTTTTGCTTCT CACTCTTTAC	436
Clone 484	Forward	P3490	TGTAGACGCAGCTATGGCTACATTAAACG AAAAAGCTGTA	437
Clone 484	Reverse	P3491	TCGTTTAATGTAGCCATAGCTGCGTCTACA TATTTGAATT	438
Clone 486	Forward	P3492	AAGAATTGAAAAAAGACTCGAGCGTCGCT TACGTTGAAG	439
Clone 486	Reverse	P3493	AAGCGACGCTCGAGTCTTTTTTCAATTCTT	440

			TTACAGCT	
Clone 488	Forward	P3494	GCGTTAATCTTTACGGCCACGATGGCGTT CGGCAGCACAT	441
Clone 488	Reverse	P3495	GAACGCCATCGTGGCCGTAAAGATTAACG CTAAAGCAAAC	442
Clone 734	Forward	P3456	GTGCAAAAGCAATTCGATTATGTAGACGC AGCTTCAGCTA	443
Clone 734	Reverse	P3457	TGCGTCTACATAATCGAATTGCTTTTGCAC TTTCCCGCCT	444

[0137] Amplification, ligation and transformation were performed as described in Example 1. Three clones for each combination of mutations were screened for AAPF activity using a 96-well plate assay as described in Example 1. Results for relative production of FNA (SEQ ID NO:9) processed from full-length FNA protein comprising a combination of mutations in pre-pro polypeptide relative to the production of FNA processed from wild-type full-length FNA are shown in Tables 5-10.

TABLE 5

Effect of combining the S49C substitution with a second mutation in the pre-pro region of FNA on the production of the mature protein

Clone No.	First mutation (clone 353)	Relative activity of First mutation to unmodified(% mean±S.D.)	Second mutation	Relative activity of the Second mutation to unmodified (% mean±S.D.)	Relative Activity of both mutations to unmodified (% mean±S.D.)
832	S49C	393.59±27.48	488(p.T19_M20insAT	9.13±5.39	100.97±24.1
687	S49C	393.59±27.48	524(p.F22_G23del)	40±10.88	105.02±38.1
713	S49C	393.59±27.48	480(S24T)	75.76±35.24	475.29±64
736	S49C	393.59±27.48	541(p.G32_K33insG)	50.01±13.55	78.57±31.4
818	S49C	393.59±27.48	734(K72D)	89.42±67.68	211.71±62.1
814	S49C	393.59±27.48	484(S78M)	90.3±74.44	43.56±23.4
634	S49C	393.59±27.48	466(S78V)	35.04±21.2	60.2±37.2
659	S49C	393.59±27.48	470(K91A)	43.61±27.77	66.37±7.57
731	S49C	393.59±27.48	486(P93S)	118.72±14.45	227.34±45.3

TABLE 6

Effect of combining the K91C substitution with a second mutation in the pre-pro region of FNA on the production of the mature protein

Clone No.	First mutation (clone 470)	Relative activity of First mutation to unmodified (% mean±S.D.)	Second mutation	Relative activity of the Second mutation to unmodified(% mean±S.D.)	Relative activity of both mutations to unmodified (% mean±S.D.)
656	K91A	43.61±27.77	488(p.T19_M20insAT	9.13±5.39	92.47±46.66
688	K91A	43.61±27.77	524(p.F22_G23del)	40.00±10.88	157.25±63.06
650	K91A	43.61±27.77	480(S24T)	75.76±35.24	118.35±64.56
783	K91A	43.61±27.77	541(p.G32_K33insG)	50.01±13.55	41.77±11.24
591	K91A	43.61±27.77	515(M48I,p.S49del)	229.68±29.83	101.97±39.49
659	K91A	43.61±27.77	353(S49C)	393.59±27.48	66.37±7.57
648	K91A	43.61±27.77	475(S49A)	33.23±24.00	117.68±53.42
606	K91A	43.61±27.77	521(S52H)	69.06±33.01	78.91±53.90
636	K91A	43.61±27.77	469(p.K57del)	7.70±2.50	132.49±9.07
672	K91A	43.61±27.77	734(K72D)	89.42±67.68	125.26±9.14
654	K91A	43.61±27.77	484(S78M)	90.30±74.44	68.11±6.26
752	K91A	43.61±27.77	466(S78V)	35.04±21.20	96.52±33.49

TABLE 7

Effect of combining the S49A substitution with a second mutation in the pre-pro region of FNA on the production of the mature protein

Clone No.	First mutation (clone 475)	Relative activity of First mutation to unmodified FNA (% mean±S.D.)	Second mutation	Relative activity of the Second mutation to unmodified FNA (% mean±S.D.)	Relative activity of both mutations to unmodified FNA (% mean±S.D.)
698	S49A	33.23±24.00	462(p.R2_S3insT)	134.20±68.40	100.86±30.28
803	S49A	33.23±24.00	488(p.T19_M20insAT	9.13±5.39	108.62±42.45
802	S49A	33.23±24.00	524(p.F22_G23del)	40.00±10.88	41.69±19.56
826	S49A	33.23±24.00	480(S24T)	75.00±19.10	77.91±19.13
785	S49A	33.23±24.00	541(p.G32_K33insG)	50.01±13.55	140.11±20.88
660	S49A	33.23±24.00	734(K72D)	89.42±67.68	93.72±18.89
827	S49A	33.23±24.00	484(S78M)	90.30±74.44	102.74±43.80
624	S49A	33.23±24.00	466(S78V)	35.04±21.20	105.01±34.43
648	S49A	33.23±24.00	470(K91A)	43.61±27.77	117.68±53.42
703	S49A	33.23±24.00	486(P93S)	118.72±14.45	272.32±45.15

TABLE 8

Effect of combining the p.T19_M20insAT insertion with a second mutation in the pre-pro region of FNA on the production of the mature protein

Clone No.	First mutation (clone 488)	Relative activity of First mutation to unmodified FNA(% mean±S.D.)	Second mutation	Relative activity of the Second mutation to unmodified FNA (% mean±S.D.)	Relative activity of both mutations to unmodified FNA (% mean±S.D.)
811	p.T19_M20insAT	9.13±5.39	448(wt)	134.20±68.40	55.77±20.57
567	p.T19_M20insAT	9.13±5.39	541(p.G32_K33insG)	50.01±13.55	70.06±35.51
601	p.T19_M20insAT	9.13±5.39	515(M48I,p.S49del)	229.68±29.83	183.98±9.97
832	p.T19_M20insAT	9.13±5.39	353(S49C)	393.59±27.48	100.97±24.08
803	p.T19_M20insAT	9.13±5.39	475(S49A)	33.23±24.00	108.62±42.45
616	p.T19_M20insAT	9.13±5.39	521(S52H)	69.06±33.01	91.57±56.34
647	p.T19_M20insAT	9.13±5.39	469(p.K57del)	7.70±2.50	93.14±41.92
669	p.T19_M20insAT	9.13±5.39	734(K72D)	89.42±67.68	110.65±33.54
725	p.T19_M20insAT	9.13±5.39	484(S78M)	90.30±74.44	280.25±69.52
632	p.T19_M20insAT	9.13±5.39	466(S78V)	35.04±21.20	42.16±20.03
656	p.T19_M20insAT	9.13±5.39	470(K91A)	43.61±27.77	92.47±46.66
829	p.T19_M20insAT	9.13±5.39	486(P93S)	118.72±14.45	157.29±68.38

TABLE 9

Effect of combining the p.F22_G23del deletion with a second mutation in the pre-pro region of FNA on the production of the mature protein

Clone No.	First mutation (clone 524)	Relative activity of First mutation to unmodified FNA (% mean±S.D.)	Second mutation	Relative activity of the Second mutation to unmodified FNA (% mean±S.D.)	Relative activity of both mutations to unmodified FNA (% mean±S.D.)
823	p.F22_G23del	40.00±10.88	462(p.R2_S3insT)	44.30±23.62	114.90±17.24
821	p.F22_G23del	40.00±10.88	448(wt)	134.20±68.40	52.87±11.04
687	p.F22_G23del	40.00±10.88	353(S49C)	393.59±27.48	105.02±38.09
802	p.F22_G23del	40.00±10.88	475(S49A)	33.23±24.00	41.69±19.56
759	p.F22_G23del	40.00±10.88	484(S78M)	90.30±74.44	58.79±15.06
692	p.F22_G23del	40.00±10.88	466(S78V)	35.04±21.20	121.46±44.94
688	p.F22_G23del	40.00±10.88	470(K91A)	43.61±27.77	157.25±63.06
684	p.F22_G23del	40.00±10.88	486(P93S)	118.72±14.45	812.67±46.20

TABLE 10

Effect of combining the P93S substitution with a second mutation in the pre-pro region of FNA on the production of the mature protein

Clone No.	First mutation (clone 486)	Relative activity of First mutation to unmodified FNA (% mean±S.D.)	Second mutation	Relative activity of the Second mutation to unmodified FNA (% mean±S.D.)	Relative activity of both mutations to unmodified FNA (% mean±S.D.)
829	P93S	118.70±14.50	p.T19_M20insAT	9.10±5.40	157.30±68.40
684	P93S	118.70±14.50	p.F22_G23del	40.00±10.90	812.20±46.20
710	P93S	118.70±14.50	S24T	75.80±35.20	299.00±76.00
564	P93S	118.70±14.50	p.G32_K33insG	50.00±13.60	163.30±53.40
599	P93S	118.70±14.50	M48I, p.S49del	229.70±29.80	258.20±48.50
731	P93S	118.70±14.50	S49C	393.60±27.50	227.30±45.30
703	P93S	118.70±14.50	S49A	33.20±24.00	272.30±45.20
615	P93S	118.70±14.50	S52H	69.10±33.00	157.40±68.70
644	P93S	118.70±14.50	pK57del	7.70±2.50	167.00±43.30
666	P93S	118.70±14.50	K72D	89.40±67.70	187.10±28.30
722	P93S	118.70±14.50	S78M	90.30±74.40	217.00±39.50
631	P93S	118.70±14.50	S78V	35.00±21.20	161.00±38.30

[0138] The data show that the majority of combinations resulted in a relative AAPF activity that was greater than that obtained as a result of individual mutations i.e. most combinations of mutations had a synergistic effect on the AAPF activity.

[0139] All *B. subtilis* cells expressing a full-length FNA comprising a pre-pro polypeptide having a combination of mutations had a level of production of the mature FNA that was greater than that of the *B. subtilis* cells that expressed the wild-type pre-pro-FNA.

[0140] The majority of *B. subtilis* clones expressing a full-length FNA comprising a pre-pro polypeptide having a combination of mutations had a greater level of production of the mature FNA than clones expressing produced a full-length FNA comprising a pre-pro polypeptide having a single mutation.

EXAMPLE 3

[0141] Site Evaluation Libraries (SELs) were constructed to generate positional libraries at each of the first 103 amino acid positions that comprise the pre-pro region of FNA. Site saturation mutagenesis of the pre-pro sequence of the full-length FNA protease was performed to identify amino acid substitutions that increase the production of FNA by a bacterial host cell.

SEL Library Construction

[0142] Pre-Pro-FNA SEL production was performed by DNA 2.0 (Menlo Park, CA) using their technology platform for gene optimization, gene synthesis and library generation under proprietary DNA 2.0 know how and/or intellectual property. The pAC-FNA10 plasmid containing the full-length FNA polynucleotide (GTGAGAAGCAAAAAATTGTGGATCAGTTTGCTGTTTGCTTTAGCGTTAATCTTTACGATGGCGTT CGGCAGCACATCCAGCGCGCAGGCGGCAGGGAAATCAAACGGGGAAAAGAAATATATTGTCGG GTTTAAACAGACAATGAGCACGATGAGCGCCGCTAAGAAGAAAGATGTCATTTCTGAAAAAGGC GGGAAAGTGCAAAAGCAATTCAAATATGTAGACGCAGCTTCAGCTACATTAAACGAAAAAGCTGT AAAAGAATTGAAAAAAGACCCGAGCGTCGCTTACGTTGAAGAAGATCACGTAGCACACGCGTAC GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTACA CTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGATTCTTCTCATCCTGATTTAAAG GTAGCAGGCGGAGCCAGCATGGTTCCTTCTGAAACAAATCCTTTCCAAGACAACAACTCTCACG GAACTCACGTTGCCGGCACAGTTGCGGCTCTTAATAACTCAATCGGTGTATTAGGCGTTGCGCC AAGCGCATCACTTTACGCTGTAAAAGTTCTCGGTGCTGACGGTTCCGGCCAATACAGCTGGATC ATTAACGGAATCGAGTGGGCGATCGCAAACAATATGGACGTTATTAACATGAGCCTCGGCGGAC CTTCTGGTTCTGCTGCTTTAAAAGCGGCAGTTGATAAAGCCGTTGCATCCGGCGTCGTAGTCGTT GCGGCAGCCGGTAACGAAGGCACTTCCGGCAGCTCAAGCACAGTGGGCTACCCTGGTAAATAC CCTTCTGTCATTGCAGTAGGCGCTGTTGACAGCAGCAACCAAAGAGCATCTTTCTCAAGCGTAG GACCTGAGCTTGATGTCATGGCACCTGGCGTATCTATCCAAAGCACGCTTCCTGGAAACAAATAC GGCGCGTTGAACGGTACATCAATGGCATCTCCGCACGTTGCCGGAGCGGCTGCTTTGATTCTTT CTAAGCACCCGAACTGGACAAACACTCAAGTCCGCAGCAGTTTAGAAAACACCACTACAAAACTT GGTGATTCTTTCTACTATGGAAAAGGGCTGATCAACGTACAGGCGGCAGCTCAGTAA; SEQ ID NO:2) was sent to DNA 2.0 for the generation of the SELs. A request was made to DNA 2.0 to generate positional libraries at each of the 107 amino acids of the pre-pro region of FNA (Figure 1). For each of the 107 sites shown enumerated in Figure 1, DNA 2.0 provided no less than 15 substitution variants at each of the positions. These gene constructs were obtained in 96 well plates each containing 4 single position libraries per plate. The libraries consisted of transformed *B. subtilis* host cells (genotype: *ΔaprE, ΔnprE, ΔspoIIE, amyE::xylRPxylAcomK-phleo*) that had been transformed with expression plasmids encoding the FNA variant sequences. These cells were

received as glycerol stocks plated in 96 well plates, and the polynucleotide encoding each variant was sequenced, and the activity of the encoded variant protein was determined as described above. Individual clones were cultured as described in Example 1 in order to obtain the different FNA protein variants for functional characterization. FNA production is reported in Table 11 as the ratio of production of FNA processed from full-length FNA protein comprising mutated pre-pro polypeptides relative to the production of FNA processed from wild-type full-length FNA at a given position.

TABLE 11

Effect of mutations in the pre-pro region of FNA on the production of the mature protein

Position	Original residue	Variant amino acids																			
		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
1	V																				
2	R		0.57	0.93	0.27	**1.19**		0.23	0.64	0.46	0.25	0.47	**1.02**	**1.03**	0.15	0.40	0.44	0.71			**1.67**
3	S	**1.00**	0.78	0.81	0.97	0.32	0.33	0.27			0.56	1.04		**1.06**	0.67	**1.49**	1.13		0.68	0.87	0.85
4	K	0.02	0.60	0.49	-0.04	0.27	0.32		0.51	0.60	0.57	-0.01	0.47	0.58		-0.02	0.41		-0.04	-0.02	0.37
5	K	0.00	0.00		0.20	0.39	0.40			1.26	0.25	0.34	0.71	0.10		0.53	0.75	0.88	0.89	0.38	
6	L	0.38	0.88		0.80	0.37	0.83	0.43	0.44	**1.17**	0.82	**1.03**	0.46	0.34		0.34	0.83	0.59	0.80		0.69
7	W	0.46	0.37	0.38	**1.05**	0.32	0.26		0.47		0.28	0.72		0.54		0.35	0.86	0.71	0.76	0.92	
8	I	0.48	0.02	0.19	0.41	0.46	0.80		1.04	0.03	0.70		0.01	0.53	-0.02	0.01	0.39	-0.06	0.43	1.05	0.29
9	S	0.98	0.58	0.44	0.12	0.58	0.22		0.47	0.59	0.24	0.44		0.54		0.60	0.57	0.38	0.72	0.33	
10	L	**1.10**	**1.24**	0.00	0.01	0.03	**1.15**	0.43	-0.01	0.25	0.86	**1.14**	0.00	0.83	0.61	0.31	0.80	**1.73**	0.87	0.73	0.00
11	L	**1.04**	0.00		0.44	**1.26**	0.75	0.73	0.68	0.66	1.16	0.61	0.67	0.60	0.67	0.60	0.95	**1.24**	0.86	0.00	0.68
12	F	0.67	**1.07**	0.11	-0.13	0.90	0.39	0.44		0.16	0.77			**1.05**	0.51	0.12	0.79	**1.00**	0.86	0.73	0.38
13	A	0.95	**1.20**	0.42		0.77	**1.47**			0.80	0.70	0.86	0.42	0.36	0.79	0.35	**1.22**	0.42	0.94	0.37	0.16
14	L		0.30	0.12	0.00	**1.49**		0.62	0.95	0.15	-0.01		0.55	0.55	0.60	0.04	0.41	0.47	0.50	0.61	0.22
15	A	0.38	0.56		0.36	0.38	**1.05**		0.61	0.14	0.45	**1.23**	0.53	0.42	0.43	-0.02	0.44	**1.03**	**1.28**	0.29	
16	L		0.57		0.17	0.91	0.53	0.37	0.85		0.41	0.45	0.24	0.32	0.54	-0.04	0.48		**1.21**	0.37	
17	I	0.46	0.52	0.24	0.31	0.45	0.67	0.34	0.34		0.64		0.28	0.30	0.42	-	1.25		0.56	0.29	

Position	Original residue	Variant amino acids																			
		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
																0.04					
18	F	0.56	0.84	0.06	0.27	0.37	0.63		0.72	0.04	0.75	0.47	0.22	0.44	0.44	0.09	0.42	0.47	0.51	0.31	0.38
19	T	0.54	0.49	0.42		0.55	0.73		0.68		0.46	0.48		**1.01**	0.63	0.14	**1.36**	0.22	0.71	0.40	
20	M	0.57	0.72	0.38	0.65	0.78	0.53	0.60	0.93		0.48	0.83		0.40		0.34	0.51	0.84	**1.06**	0.53	0.88
21	A	0.92	0.53	0.48	0.52	0.62	0.25			-0.02	0.48	0.55	0.13	0.60	0.12	0.17	**1.07**	0.51	0.56	0.33	
22	F	0.43	0.43		**1.23**	0.37	0.41	0.66	0.55	0.60	0.73	0.41	0.72	0.19	0.43	0.42	0.48	0.47	0.51	0.39	0.50
23	G	0.55	0.78			**1.33**	1.09	0.41	0.47		0.47			0.56	**1.21**	0.67	0.58	0.50	0.66	**1.50**	0.45
24	S		0.67		0.61	0.61	0.82	0.29			0.55	0.59	0.71	0.82	0.89	0.34	0.92		**1.61**	0.67	0.48
25	T	**1.12**	0.58	**1.32**	0.61	0.52	0.59	0.49	0.79		0.64	0.55		0.40		0.31	0.84	0.60	0.76	**1.15**	0.43
26	S	0.81	**1.35**	0.79	0.69	0.01	0.81	**1.36**	0.64	0.37	0.41	0.73	0.65		0.75	0.47	0.25	0.63	0.75	0.71	0.75
27	S	**1.06**	0.63		0.89	**1.76**	0.31	**1.86**	0.96		0.90	0.64		**3.23**		0.72	0.80	**1.07**	**2.04**	0.66	**1.03**
28	A	0.98			0.57	0.80	0.68		0.81	0.38	0.83	0.66	0.97	0.49	0.56	0.35	0.88	0.87	**1.14**	0.50	
29	Q	0.61	0.51		**1.22**	0.93	0.86		**1.15**		0.91	0.54		0.40	0.49	**1.18**	**1.45**	**1.47**	0.62	0.64	
30	A	0.81	**1.13**	0.97	0.61	0.98	0.47	0.97		0.35	0.54	0.66	0.51		0.49	0.93	0.29	0.72	0.88	0.81	0.62
31	A	1.06	0.49	0.29	0.56	0.27	0.63	**1.39**	0.49	**1.45**	0.49	0.95	**2.60**	0.37	0.19	0.49	**1.80**	-0.01	**1.17**	**1.12**	
32	G	0.94	**1.41**	0.61	0.92	**1.30**	0.56		0.52		0.73	**1.05**	**1.68**	**1.11**		0.90	**1.14**		**1.19**	-0.02	0.85
33	K	0.41	0.51	0.42	**1.07**	**1.33**	0.76		0.77	0.23	-0.02	**1.04**		**1.17**		0.55	**1.23**		0.12	0.30	0.21
34	S	0.64	0.98	**1.18**	0.83	0.50	0.89	**1.08**		0.57	0.38	0.84	0.56	**1.02**	0.53	0.76	0.65	0.54	**1.41**	0.55	0.72
35	N	0.75	**1.47**		0.43	0.63	0.71		0.72		0.14	0.37	0.50	0.98	**1.18**	0.91	**1.39**	0.03	0.57	0.19	0.79
36	G	0.68	**1.20**	**1.68**	0.50	0.73	1.40		0.49		0.78	0.58		0.51	0.59	0.47	**1.25**	0.60	0.59	**1.17**	**1.97**
37	E	0.95	**1.20**	0.64	0.54	0.66	**1.29**		0.85	**1.39**	0.44	0.52	0.16	0.00	**1.09**	0.28	0.59	0.35	0.98	0.87	0.39
38	K	0.25	0.60	0.03	1.17	**1.30**	0.60	0.57	0.51	0.99	0.57	0.20	0.97		**1.13**	0.48	**1.03**	0.86	0.67	**1.14**	0.99

Position	Original residue	Variant amino acids																			
		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
39	K	**1.21**	**1.03**		0.84		**1.11**	0.87	**1.25**		**2.64**	**1.00**		**1.35**	0.94	0.82	**1.17**	**1.17**	**1.27**	0.77	0.49
40	Y	0.41	0.82		0.22	0.04	0.16	0.14	0.39		0.22	0.75		-0.05	0.13	0.07	0.10		0.59	0.82	0.36
41	I	0.03	0.15	-0.03	0.03	0.64	0.06		0.71		0.54	0.68		-0.03	0.05	-0.04	0.06		0.55		-0.03
42	V	-0.03	0.31	-0.02		0.03	-0.03	-0.02			0.22	0.06		0.02	-0.02	0.03	-0.03	0.06	0.49	0.00	0.02
43	G	0.00		0.01	0.00		0.01		0.00	0.00	0.00	0.26		0.00		0.00	0.19	0.00	-0.01	0.00	0.00
44	F	0.46	0.06	0.22		0.65	0.25		0.27		0.49	0.05		-0.07	0.06	0.20	0.58	0.49	0.42	0.58	
45	K	0.62	0.40		0.65	0.70	**1.56**				0.48	0.74		0.34	0.53	0.96	**1.14**	0.51	0.59	0.25	0.83
46	Q	0.48	0.59	0.37		0.63	0.46		0.64		0.54	0.48			0.60	0.49	**1.27**	0.56	0.48	0.42	0.46
47	T	0.13		0.56	**1.31**	**1.43**	0.52	-0.03		0.58	0.37	0.43		0.66	0.51	0.94	0.53	1.16	0.48		
48	M	-0.02	0.60	0.02	-0.11	0.00	**1.42**		**1.46**	0.45	0.76	0.53				0.43	0.42	**1.54**	0.48	**1.38**	**2.55**
49	S	0.60	0.47		**1.08**	0.55	0.60		0.04	0.62	-0.06	0.31	0.47	0.03	0.03	0.81	0.68	-0.02	0.58		-0.04
50	T	0.98	0.97	**1.15**	0.70	0.83	0.45	0.68	0.43		0.96	0.94			0.65	0.15	0.68	0.74	0.91		**1.09**
51	M	**1.37**	0.74	0.76	0.73	0.46	0.81	**1.07**	0.75	0.91	0.72	0.52	0.79	0.78	0.34	0.79	0.61	0.73	0.59	0.58	0.55
52	S	**2.67**			0.85		0.97		**1.31**	0.89	0.41	**1.06**		0.72	0.67	0.95	0.55	0.95	0.45	0.95	0.85
53	A	0.91	0.56	**1.11**	**1.64**	0.68	0.88		0.55	0.57	0.59	**1.02**	0.72	0.68	**1.50**	0.64	0.67	**1.26**	0.80	0.50	0.46
54	A	0.55	0.46	0.98	0.54	**1.26**	**1.08**	**0.04**	**1.19**	0.08	0.57			0.96	0.99	0.80	**1.04**	0.71	0.89	0.43	
55	K	0.86		**1.01**		0.60	0.90		0.47	0.73	0.54	0.73	0.75		0.11	0.50	0.75	0.75		0.42	0.64
56	K				0.98	0.50	0.83		0.86	0.43	0.51	0.81		0.39	0.46	0.82	0.60	0.80	0.27	0.54	0.72
57	K		0.69	0.54	**1.55**	0.50	0.84		0.42	0.19	0.75	0.48	**1.06**		0.62	**1.37**	0.10	0.42	0.52	0.44	0.15
58	D	**1.21**	**1.02**	0.66	**1.30**	**1.04**	**1.35**		0.92	**2.25**	0.82	0.94	0.46			**1.45**	**1.13**	**1.06**	0.79	**1.05**	0.44

Position	Original residue	Variant amino acids																			
		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
59	V	0.43	0.64	0.46	**1.12**	0.63	0.43		0.71	0.51	0.44		0.73		0.54	0.80	0.57	0.52	0.60	0.36	0.50
60	I	0.13		0.05	0.05	0.19	0.13		0.32	0.00	0.31	0.65	0.05		0.07	0.08	0.08	0.09	0.43	0.08	0.07
61	S	**1.07**	0.41	0.97	0.57		0.65		**1.13**	0.26	0.51			0.46		**1.41**	1.25	0.51	0.62	0.47	0.80
62	E	**1.07**	0.81	0.76	0.71	0.53	**1.21**	**1.07**	0.52	0.54	0.40		**1.38**	0.15	0.66	0.81	**1.92**	**1.40**	0.50	0.77	
63	K	**1.13**	**1.19**	-0.08	**1.45**	**1.60**	**1.36**		0.83	0.72	0.91	0.04	**1.12**		**1.27**	**1.73**	0.73	**1.05**	0.86	0.44	0.41
64	G	0.32		**1.22**		0.54	1.13		0.24	0.71	0.03	**1.07**		0.26	**1.20**	0.56	**1.31**		0.42	0.17	0.50
65	G	0.05		0.06	0.06	0.25	0.55	0.13	0.49	0.02	0.44	0.14		0.04	0.16	0.09	0.17	0.11	0.59	0.25	0.25
66	K			0.62	**1.03**		0.33	0.67	0.17	0.18	0.15	0.79		0.14	0.57	0.79	0.60	0.44	0.56		0.45
67	V		0.60	0.96	0.57	0.85	**1.44**	0.30	0.61	0.45	**1.17**	0.56	0.65	0.79	0.93	0.50	0.78	0.65	0.97	0.27	0.47
68	Q	0.52	**1.55**	**1.05**		0.53	0.67	0.56	0.47	0.31	0.74	0.52	0.58	0.51	0.53	**1.24**	0.97	0.87			0.70
69	K	0.74	0.44	0.30	0.69	0.66	0.42	0.57	0.93	0.42	0.49	0.48	0.60	0.18		0.70	0.74	0.48	0.49		**1.40**
70	Q	0.98	0.49		**1.01**		0.60	0.43	0.54	**1.11**	0.80	0.18	0.67	**1.29**	0.67	0.28	**1.48**	0.63	**1.37**	0.57	0.46
71	F	0.11	0.15	0.03	0.03	0.15	0.08	0.11	0.00	0.02	0.41	0.72	0.13	0.03	-0.07	0.04	0.03	0.07	0.07	0.26	0.76
72	K	0.50	0.70	0.50	0.28		0.98	0.09	0.81	0.66	0.71	0.47	0.58	0.60	0.00	0.32	0.54	0.65	0.10	0.79	0.22
73	Y	0.45	0.74	0.42	0.65	0.60	0.28	0.50	0.63	0.25	0.53	0.52	0.09	-0.11	0.90	0.35	0.49	0.25	0.55	0.74	0.82
74	V	0.53	**1.82**	0.22	0.65	0.56	0.22	0.12		-0.05	0.58		0.18		0.68	0.02	0.55	0.50	0.66	0.15	0.14
75	D	0.58		0.33	0.73		**1.22**	0.55	0.43	0.50	0.92	0.62	0.67	0.76	0.40	0.63	0.62	0.61	0.80	0.54	
76	A	0.66	0.36		0.18		0.62	0.08		0.06	0.21	0.96		-0.01	0.69	0.04	0.44	0.06	**2.62**	-0.02	0.00
77	A	1.15	0.74	0.66	**1.26**	0.63	0.38	0.48		0.47	0.02	0.79	0.44	0.02	0.67	0.37	0.36	0.70	**2.43**		**1.54**
78	S		0.68	0.52		0.92	0.78	0.53		0.99	0.95	0.39	0.47	0.98	**1.19**	0.68	0.88		0.56	0.40	0.60
79	A	0.89	0.94	0.03	0.07	0.38	0.50	0.03	0.61	0.02	0.48	0.45	0.01	-0.02	0.02	0.04	0.58	0.62	0.66	0.08	0.07

Position	Original residue	Variant amino acids																			
		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
80	T		0.90	**1.09**	0.72		0.57	0.79	0.83	0.48	**1.22**		**1.01**	0.09	0.93	0.80	0.78	0.89	0.85	0.71	0.79
81	L	0.56	0.79	0.09	0.04		0.11	0.02		0.03	0.81		0.02	0.14	0.04	0.02	0.14	0.07	0.35	0.07	
82	N	0.62	**1.09**	**1.05**	0.68		0.97			0.86	0.33	0.85	0.42	**1.00**	**1.22**	0.99	**1.06**	**1.25**	0.62	0.34	0.31
83	E	0.60		0.09	0.44		**1.49**	0.56	0.94	0.52			**1.02**	0.57	0.79	0.49	0.46	0.59	0.59	0.30	
84	K	0.97	0.44	0.44	0.51		0.54	0.85		0.47		**1.33**		0.23	0.61	0.72	0.56	0.50	0.47		0.71
85	A	0.13	0.57		0.60	0.51	0.62		0.48	0.41	0.40		0.49	0.15	0.19	0.13	0.52	0.63	0.50		0.33
86	V	0.59		0.25	0.95	0.57	0.37		0.97	0.81	0.84	0.63	0.33	0.05		0.41	0.53	0.58		0.63	0.55
87	K	0.54		0.98	0.09	0.40	0.52			2.22	0.20	0.47	0.29	0.67		**1.08**	0.48	0.51	0.95	0.43	0.50
88	E	**1.02**	0.49	**1.09**			**1.98**	0.64	0.43	0.53	0.49		0.96	0.16		0.72	0.14	**1.13**	**1.74**	0.42	
89	L	0.21	0.47	0.03	0.09	0.18	0.10		0.02	-	-		-	-						-	-
90	K	0.90		**1.01**	0.60	0.57	0.51	0.68	0.80	0.55	0.10		0.01	0.02	0.03	0.02		0.41	**1.11**	0.07	0.09
91	K	0.52		0.53	0.05	0.67	0.23	0.90		0.55	0.41	0.43	0.45	0.02	0.53	0.55		0.98	0.66		0.33
92	D	0.47		**3.51**	**1.13**	0.44	0.28	0.57	0.61	0.16	0.67	0.49	0.52		0.71	0.22	**1.22**	0.74	0.90	0.36	0.05
93	P	0.78	0.77	0.76	0.80		**1.10**			0.44	0.46	0.49	**1.33**	0.64	0.69	0.80		0.83	0.70	0.78	
94	S	0.57		0.64	0.71		0.60		0.89	0.84	0.82	0.78	0.29	0.44	0.68	0.47	0.62	0.62	0.67		
95	V	0.19		0.03	0.03		0.04	0.03	0.55	0.03	0.35	0.15	0.03	0.02	0.03	0.02	0.04	0.40	0.14		
96	A	0.82		0.49	0.09	0.36	**1.11**		0.57	0.77	0.89	0.46	**1.25**	0.04	0.58	0.56	0.93	**1.23**	0.46		0.36
97	Y	0.17	0.16	0.12	0.15		0.06	0.15		0.11		0.17	0.05	0.11	0.19	0.27	0.25	0.25	0.18	0.93	
98	V		0.53	0.02	0.07	0.11	0.02	0.02	0.38	0.02	0.93		0.01	0.01	0.05	0.02	0.02	0.28	0.61	0.02	
99	E	0.32	0.23	0.38	0.57		0.16	0.21		0.05	0.11	0.09	0.16	0.03	0.53	0.39		0.19	0.12	0.03	

Position	Original residue	Variant amino acids																			
		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
100	E	0.69		0.73	0.78	0.42	0.75	0.39		0.46	0.67	0.41	0.11	0.70	0.63	0.20	0.43	0.75	0.43	0.69	
101	D	0.10	-	0.28	0.14		0.03	0.14	-	0.03	0.03	0.06	0.23		0.02	0.02	0.03	0.08	0.06	0.03	0.02
102	H	0.57		0.83	0.62		0.42	0.98		0.96	0.24	0.39		0.06	0.63	0.73	0.90	**1.13**	0.97	0.96	
103	V	0.03		0.90	0.02	0.90	0.01	0.54	0.55	0.53	0.07	0.04	0.74	0.04	0.07	0.03		0.03	0.05		

EXAMPLE 4

Production of protease from Bacillus subtilis having stably integrated constructs encoding modified proteases

[0143] Enhanced production of protease in *Bacillus subtilis* when expressed from a replicating vector pAC-FNA10 was confirmed when the vector was integrated into the chromosome of *Bacillus subtilis* using the pJH integrating vector (Ferrari et al. J. Bacteriol. 154:1513-1515 [1983]).

[0144] For vector integration, the upstream region of AprE promoter was added to the short promoter present in pAC-FNA10 by extension PCR. For this purpose, two fragments were amplified-one using the pJH-FNA plasmid (Figure 6) as the template and the other using the pAC-FNA10 plasmid with a chosen mutation in the pre-pro region of FNA as template. The first fragment, containing the missing upstream region of the AprE promoter, was amplified from the pJH-FNA plasmid using primers P3249 and P3439 (Table 12). The second fragment, spanning the short aprE promoter, modified pre-pro and mature FNA region as well as transcription terminator was amplified by primers P3438 and P3435 (Table 12) using the pAC-FNA10 with the chosen modified pre-pro as template. These two fragments contained an overlap, which allowed to recreate the full-length aprE promoter (with FNA and terminator) by mixing both fragments together and amplifying with the flanking primers containing EcoRI and BamHI restriction sites (P3255 and P3246; Table 12). The resulting fragment containing the full-length aprE promoter, modified pre-pro region, mature FNA region and the transcription terminator was digested by EcoRI and BamHI and ligated with pJH-FNA vector, which was also digested by the same restriction enzymes. Similarly, a control fragment containing the full-length aprE promoter, the unmodified sequence encoding the unmodified parent pre-pro region and mature FNA region, and the transcription terminator was created (SEQ ID NO:452). The pJH-FNA construct containing DNA encoding the control unmodified or a modified protease was transformed into *Bacillus subtilis* strain (genotype *ΔaprE, ΔnprE, spoIIE, amyE::xylRPxylAcomK-phleo*) and cultured as described in Example 1. AAPF activity of the mature FNA proteases produced when processed from a modified full-length FNA was determined and quantified as described in Example 1, and its production was compared to that of the mature FNA processed from the unmodified full-length FNA.

[0145] The sequence of the long aprE promoter is set forth as SEQ ID NO:445

AATTCTCCATTTTCTTCTGCTATCAAAATAACAGACTCGTGATTTTCCAAACGAGCTTTCAAAA
AAGCCTCTGCCCCTTGCAAATCGGATGCCTGTCTATAAAATTCCCGATATTGGTTAAACAGC
GGCGCAATGGCGGCCGCATCTGATGTCTTTGCTTGGCGAATGTTCATCTTATTTCTTCCTCC
CTCTCAATAATTTTTTCATTCTATCCCTTTTCTGTAAAGTTTATTTTTCAGAATACTTTTATCATC

ATGCTTTGAAAAAATATCACGATAATATCCATTGTTCTCACGGAAGCACACGCAGGTCATTTG
AACGAATTTTTTCGACAGGAATTTGCCGGGACTCAGGAGCATTTAACCTAAAAAAGCATGAC
ATTTCAGCATAATGAACATTTACTCATGTCTATTTTCGTTCTTTTCTGTATGAAAATAGTTATTT
CGAGTCTCTACGGAAATAGCGAGAGATGATATACCTAAATAGAGATAAAATCATCTCAAAAAA
ATGGGTCTACTAAAATATTATTCCATCTATTACAATAAATTCACAGAATAGTCTTTTAAGTAAG
TCTACTCTGAATTTTTTTAAAAGGAGAGGGTAAAGA (SEQ ID NO:445)

Table 12

Primers used for production of stably integrated constructs

PRIMER NAME	PRIMER SEQUENCE	SEQ ID NO:
P3249	GCGCGCGTAATACGACTCAC	446
P3439	ATTTTTTTGAGATGATTTTATCTCTATTTAGGTATATCATCTC	447
P3438	TAAATAGAGATAAAATCATCTCAAAAAAATGGGTCTACTAAA	448
P3435	ATGTATCAAGATAAGAAAGAACAAG	449
P3255	GCAGGAATTCTCCATTTTCTTC	450
P3246	TTTATTTTATAAACTCATTCCCTGAT	451

[0146] The nucleotide sequence of the expression cassette comprising the unmodified parent FNA polynucleotide in the pJH-FNA vector is set forth as SEQ ID NO:452

AATTCTCCATTTTCTTCTGCTATCAAAATAACAGACTCGTGATTTTCCAAACGAGCTTTCAAAA
AAGCCTCTGCCCCTTGCAAATCGGATGCCTGTCTATAAAATTCCCGATATTGGTTAAACAGC
GGCGCAATGGCGGCCGCATCTGATGTCTTTGCTTGGCGAATGTTCATCTTATTTCTTCCTCC
CTCTCAATAATTTTTTCATTCTATCCCTTTTCTGTAAAGTTTATTTTTCAGAATACTTTTATCATC
ATGCTTTGAAAAAATATCACGATAATATCCATTGTTCTCACGGAAGCACACGCAGGTCATTTG
AACGAATTTTTTCGACAGGAATTTGCCGGGACTCAGGAGCATTTAACCTAAAAAAGCATGAC
ATTTCAGCATAATGAACATTTACTCATGTCTATTTTCGTTCTTTTCTGTATGAAAATAGTTATTT
CGAGTCTCTACGGAAATAGCGAGAGATGATATACCTAAATAGAGATAAAATCATCTCAAAAAA
ATGGGTCTACTAAAATATTATTCCATCTATTACAATAAATTCACAGAATAGTCTTTTAAGTAAG
TCTACTCTGAATTTTTTTAAAAGGAGAGGGTAAAGAGTGAGAAGCAAAAAATTGTGGATCAGT
TTGCTGTTTGCTTTAGCGTTAATCTTTACGATGGCGTTCGGCAGCACATCCTCTGCCCAGGC
GGCAGGGAAATCAAACGGGGAAAAGAAATATATTGTCGGGTTTAAACAGACAATGAGCACG
ATGAGCGCCGCTAAGAAGAAAGATGTCATTTCTGAAAAAGGCGGGAAAGTGCAAAAGCAATT
CAAATATGTAGACGCAGCTTCAGCTACATTAAACGAAAAAGCTGTAAAAGAATTGAAAAAAGA
CCCGAGCGTCGCTTACGTTGAAGAAGATCACGTAGCACATGCGTACGCGCAGTCCGTGCCT

TACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTACACTGGATCAAATGT
TAAAGTAGCGGTTATCGACAGCGGTATCGATTCTTCTCATCCTGATTTAAAGGTAGCAGGCG
GAGCCAGCATGGTTCCTTCTGAAACAAATCCTTTCCAAGACAACAACTCTCACGGAACTCAC
GTTGCCGGCACAGTTGCGGCTCTTAATAACTCAATCGGTGTATTAGGCGTTGCGCCAAGCG
CATCACTTTACGCTGTAAAAGTTCTCGGTGCTGACGGTTCCGGCCAATACAGCTGGATCATT
AACGGAATCGAGTGGGCGATCGCAAACAATATGGACGTTATTAACATGAGCCTCGGCGGAC
CTTCTGGTTCTGCTGCTTTAAAAGCGGCAGTTGATAAAGCCGTTGCATCCGGCGTCGTAGTC
GTTGCGGCAGCCGGTAACGAAGGCACTTCCGGCAGCTCAAGCACAGTGGGCTACCCTGGT
AAATACCCTTCTGTCATTGCAGTAGGCGCTGTTGACAGCAGCAACCAAAGAGCATCTTTCTC
AAGCGTAGGACCTGAGCTTGATGTCATGGCACCTGGCGTATCTATCCAAAGCACGCTTCCT
GGAAACAAATACGGCGCGTTGAACGGTACATCAATGGCATCTCCGCACGTTGCCGGAGCGG
CTGCTTTGATTCTTTCTAAGCACCCGAACTGGACAAACACTCAAGTCCGCAGCAGTTTAGAA
AACACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAGGGCTGATCAACGTACAGGC
GGCAGCTCAGTAAAACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTTATTATTTTTCTTCC
TCCGCATGTTCAATCCGCTCCATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAA
CGGCGGGTTGACCCGGCTCAGTCCCGTAACGGCCAAGTCCTGAAACGTCTCAATCGCCGCT
TCCCGGTTTCCGGTCAGCTCAATGCCGTAACGGTCGGCGGCGTTTTCCTGATACCGGGAGA
CGGCATTCGTAATCGGATCC (SEQ IDNO:452).

[0147] The cassette contains the sequence of the long AprE promoter (underlined, SEQ ID NO:445), the pre-pro region (SEQ ID NO:7) and mature regions of FNA (SEQ ID NO:(9), and a transcription terminator.

[0148] Results of FNA production processed from one of the mutants (clone 684; Table 9) are shown in Figure 7 relative to the production of FNA production processed from the unmodified full-length FNA. These data confirmed that production of protease encoded from the integrated construct containing the modified pre-pro region was enhanced compared to that produced from the unmodified pre-pro region.

CLAIMS

We claim:

1. An isolated modified polynucleotide encoding a modified full-length protease, said isolated modified polynucleotide comprising a first polynucleotide encoding the pre-pro region of said full-length protease operably linked to a second polynucleotide encoding the mature region of said full-length protease, wherein said first polynucleotide encodes the pre-pro region of SEQ ID NO:7 and is further mutated to comprise at least one mutation, wherein said at least one mutation enhances the production of said protease by a host cell.

2. The isolated modified polynucleotide of Claim 1, wherein said modified full-length protease is an alkaline serine protease derived from a wild-type or variant precursor alkaline serine protease.

3. The isolated modified polynucleotide of Claim 2, wherein said precursor alkaline serine protease is a *Bacillus subtilis*, a *Bacillus amyloliquefaciens,* a *Bacillus pumilis* or a *Bacillus licheniformis* serine protease.

4. The isolated polynucleotide of Claim 1, wherein said host cell is a *Bacillus* sp. host cell.

5. The isolated polynucleotide of Claim 4, wherein said *Bacillus* sp. host cell is a *Bacillus subtilis* host cell.

6. The isolated modified polynucleotide of any one of Claims 1-5, wherein said second polynucleotide encodes a protease having at least about 65% identity to the protease of SEQ ID NO:9.

7. The isolated modified polynucleotide of any one of Claims 1-6, wherein said second polynucleotide encodes the protease of SEQ ID NO:9.

8. The isolated modified polynucleotide of any one of Claims 1-7, wherein said first polynucleotide comprises at least one mutation encoding at least one substitution at one or more positions selected from positions 2, 3, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 57, 58, 59, 61, 62, 63, 64, 66, 67, 68, 69, 70, 72, 74, 75, 76, 77, 78, 80, 82, 83, 84, 87, 88, 89,

90, 91, 93, 96, 100, and 102, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of SEQ ID NO:7.

9. The isolated modified polynucleotide of any one of Claims 1-8, wherein said first polynucleotide comprises at least one mutation encoding at least one substitution selected from X2F, N, P, and Y; X3A, M, P, and R; X6K, and M; X7E; I8W; X10A, C, G, M, and T; X11A, F, and T; X12C, P, T; X13C, G, and S; X14F; X15G, M, T, and V; X16V; X17S; X19P, and S; X20V; X21S; X22E; X23F, Q, and W; X24G, T and V; X25A, D, and W; X26C, and H; X27A, F, H, P, T, V, and Y; X28V; X29E, I, R, S, and T; X30C; X31H, K, N, S, V, and W; X32C, F, M, N, P, S, and V; X33E, F, M, P, and S; X34D, H, P, and V; X35C, Q, and S; X36C, D, L, N, S, W, and Y; X37C, G, K, and Q; X38F, Q, S, and W; X39A, C, G, I, L, M, P, S, T, and V; X45G and S; X46S; X47E and F; X48G, I, T, W, and Y; X49A, C, E and I; X50D, and Y; X51A and H; X52A, H, I, and M; X53D, E, M, Q, and T; X54F, G, H, I, and S; X55D; X57E, N, and R; X58A, C, E, F, G, K, R, S, T, W; X59E; X61A, F, I, and R; X62A, F, G, H, N, S, T and V; X63A, C, E, F, G, N, Q, R, and T; G64D, M, Q, and S; X66E; X67G and L; X68C, D, and R; X69Y; X70E, G, K, L, M, P, S, and V; X72D and N; X74C and Y; X75G; X76V; X77E, V, and Y; X78M, Q and V; X80D, L, and N; X82C, D, P, Q, S, and T; X83G, and N; X84M; X87R; X88A, D, G, T, and V; X89V; X90D and Q; X91A; X92E and S; X93G, N, and S; X96G, N, and T; X100Q; and X102T, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

10. The isolated modified polynucleotide of any one of Claims 1-9, wherein said first polynucleotide comprises at least one mutation encoding at least one substitution selected from R2F, N, P, and Y; S3A, M, P, and R; L6K, and M; W7E; I8W; L10A, C, G, M, and T; L11A, F, and T; F12C, P, T; A13C, G, and S; L14F; A15G, M, T, and V; L16V; I17S; T19P, and S; M20V; A21S; F22E; G23F, Q, and W; S24G, T and V; T25A, D, and W; S26C, and H; S27A, F, H, P, T, V, and Y; A28V; Q29E, I, R, S, and T; A30C; A31H, K, N, S, V, and W; G32C, F, M, N, P, S, and T; K33E, F, M, P, and S; S34D, H, P, and V; N35C, Q, and S; G36C, D, L, N, S, W, and Y; E37C, G, K, and Q; K38F, Q, S, and W; K39A, C, G, I, L, M, P, S, T, and V; K45G and S; Q46S; T47E and F; M48G, I, T, W, and Y; S49A, C, E and I; T50D, and Y; M51A and H; S52A, H, I, and M; A53D, E, M, Q, and T; A54F, G, H, I, and S; K55D; K57E, N, and R; D58A, C, E, F, G, K, R, S, T, W; V59E; S61A, F, I, and R; E62A, F, G, H, N, S, T and V; K63A, C, E, F, G, N, Q, R, and T; 64D, M, Q, and S; K66E; V67G and L; Q68C, D, and R; K69Y; Q70E, G, K, L, M, P, S, and V; K72D and N; V74C and Y; D75G; A76V; A77E, V, and Y; S78M, Q and V; T80D, L, and N; N82C, D, P, Q, S, and T; E83G, and N; K84M; K87R; E88A, D, G, T, and V; L89V; K90D and Q; K91A; D92E and S; P93G, N, and S; A96G, N, and T; E100Q; and H102T, wherein the positions are

numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

11. The isolated modified polynucleotide of any one of Claims 1-10, wherein said first polynucleotide comprises at least one combination of mutations encoding a combination of substitutions selected from X49A-X24T, X49A-X72D, X49A-X78M, X49A-X78V, X49A-X93S, X49C-X24T, X49C-X72D, X49C-X78M, X49C-X78V, X49C-X91A, X49C-X93S, X91A-x24T, X91A-X49A, X91A-X52H, X91A-X72D, X91A-X78M, X91A-X78V, X93S-X24T, X93S-X49C, X93S-X52H, X93S-X72D, X93S-X78M, and X93S-X78V, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

12. The isolated modified polynucleotide of any one of Claims 1-11, wherein said first polynucleotide comprises at least one combination of mutations encoding a combination of substitutions selected from S49A-S24T, S49A-K72D, S49A-S78M, S49A-S78V, S49A-P93S, S49C-S24T, S49C-K72D, S49C-S78M, S49C-S78V, S49C-K91A, S49C-P93S, K91A-S24T, K91A-S49A, K91A-S52H, K91A-K72D, K91A-S78M, K91A-S78V, P93S-S24T, P93S-S49C, P93S-S52H, P93S-K72D, P93S-S78M, and P93S-S78V, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

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13. The isolated modified polynucleotide of any one of Claims 1-7, wherein said first polynucleotide comprises at least one mutation encoding at least one deletion selected from p.X18_X19del, p.X22_23del, pX37del, pX49del, p.X47del, pX55del and p.X57del, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

14. The isolated modified polynucleotide of any one of Claims 1-7 and 13, wherein said first polynucleotide comprises at least one mutation encoding at least one deletion selected from p.I18_T19del, p.F22_G23del, p.E37del, p.T47del, p.S49del, p.K55del, and p.K57del, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

15. The isolated polynucleotide of any one of Claims 1-7, 13 and 14, wherein said first polynucleotide comprises at least one mutation encoding at least one insertion selected from p.X2_X3insT, p.X30_X31insA, p.X19_X20insAT, p.X21_X22insS, p.X32_X33insG, p.X36_X37insG, and p.X58_X59insA, wherein the positions are numbered by correspondence

with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

16. The isolated modified polynucleotide of any one of Claims 1-7, and 15, wherein said first polynucleotide comprises at least one mutation encoding an insertion selected from p.R2_S3insT, p.A30_A31insA, p.T19_M20insAT, p.A21_F22insS, p.G32_K33insG, p.G36_E37insG, and p.D58_V59insA, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

17. The isolated polynucleotide of any one of Claims 1-7, wherein said first polynucleotide comprises at least two mutations encoding at least one substitution and at least one deletion selected from X46H-p.X47del, X49A-p.X22_X23del, x49C-p.X22_X23del, X48I-p.X49del, X17W-p.X18_X19del, X78M-p.X22_X23del, X78V-p.X22_X23del, X78V-p.X57del, X91A-p.X22_X23del, X91A-X48I-pX49del, X91A-p.X57del, X93S-p.X22_X23del, and X93S-X48I-p.X49del, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

18. The isolated modified polynucleotide of any one of Claims 1-7, and 17, wherein said first polynucleotide comprises at least two mutations encoding at least one substitution and at least one deletion selected from the group consisting of Q46H-p.T47del, S49A-p.F22_G23del, S49C-p.F22_G23del, M48I-p.S49del, I17W-p.I18_T19del, S78M-p.F22_G23del, S78V-p.F22_G23del, K91A-p.F22_G23del, K91A-M48I-pS49del, K91A-p.K57del, P93S-p.F22_G23del, and P93S-M48I-p.S49del, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

19. The isolated modified polynucleotide of any one of Claims 1-7, wherein said first polynucleotide comprises at least two mutations encoding at least one substitution and at least one insertion selected from X49A-p.X2_X3insT, X49A-p32X_X33insG, X49A-p.X19_X20insAT, X49C-p.X19_X20insAT, X49C-p.X32_X33insG, X52H--p.X19_X20insAT, X72D-p.X19_X20insAT, X78M-p.X19_X20insAT, X78V-p.X19_X20insAT, X91A-p.X19_X20insAT, X91A- p.X32_X33insG, X93S- p.X19_X20insAT, and X93S- p.X32_X33insG, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

20. The isolated modified polynucleotide of any one of Claims 1-7, and 19, wherein said first polynucleotide comprises at least two mutations encoding at least one substitution and at least one insertion selected from S49A-p.R2_S3insT, S49A-p32G_K33insG, S49A-p.T19_M20insAT,

S49C-p.T19_M20insAT, S49C-p.G32_K33insG, S49C-p.T19_M20insAT, S52H--p.T19_M20insAT, K72D-p.T19_M20insAT, S78M-p.T19_M20insAT, S78V-p.T19_M20insAT, K91A-p.T19_M20insAT, K91A- p.G32_K33insG, P93S- p.T19_M20insAT, and P93S-p.G32_K33insG, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

21. The isolated modified polynucleotide of any one of Claims 1-7, wherein said first polynucleotide comprises at least two mutations encoding at least one deletion and at least one insertion selected from p.X57del-p.X19_X20insAT, and p.X 22_X23del-p.X2_X3insT, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

22. The isolated modified polynucleotide of any one of Claims 1-7 and 21, wherein said first polynucleotide comprises at least two mutations encoding a deletion and an insertion selected from pK57del-p.T19_M20insAT, and p.F22_G23del-p.R2_S3insT.

23. The isolated polynucleotide of any one of Claims 1-7, wherein said first polynucleotide comprises at least three mutations encoding at least one deletion, one insertion and one substitution corresponding to p.X49del-p.X19_X20insAT-X48I, and wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

24. The isolated polynucleotide of any one of Claims 1-7 and 23, wherein said first polynucleotide comprises at least three mutations encoding at least one deletion, one insertion and one substitution corresponding to p.S49del-p.T19_M20insAT-M48I, wherein the positions are numbered by correspondence with the amino acid sequence of the pre-pro polypeptide of the FNA protease set forth as SEQ ID NO:7.

25. An isolated polypeptide encoded by the modified full-length polynucleotide of any one of Claims 1- 24.

26. An expression vector comprising the isolated modified polynucleotide of any one of Claims 1-24.

27. The expression vector of Claim 26, further comprising an AprE promoter.

28. A host cell comprising the expression vector of any one of Claims 26-27.

29. The host cell of Claim 28, wherein the host cell is a *Bacillus* sp. host cell.

30. The host cell of Claim 29, wherein said Bacillus sp. host cell is selected from *B. subtilis*, *B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens*, *B. clausii*, *B. halodurans*, *B. megaterium, B. coagulans, B. circulans, B. lautus*, and *B. thuringiensis*.

31. The host cell of any one of Claims 28-30, wherein said host cell is a *B. subtilis* host cell.

32. A method of producing a mature protease in a *Bacillus sp.* host cell, said method comprising:

(a) providing the expression vector of any one of Claims 26-27;

(b) transforming a host cell with said expression vector;

(c) culturing said host cell under suitable conditions such that said protease is produced by said host cell.

33. The method of Claim 32, wherein said *Bacillus* sp. host cell is a *Bacillus subtilis* host cell.

34. The method of any one of Claims 32-33, wherein said protease is an alkaline serine protease.

35. The method of any one of Claims 32-34, wherein said modified polynucleotide encodes a protease comprising a mature region that is at least 65% identical to SEQ ID NO:9.

36. The method of any one of Claims 32-35, wherein said first polynucleotide encodes the pre-pro region of SEQ ID NO:7, wherein said first polynucleotide comprises at least one mutation to increase the production of said mature region of said protease, and wherein said second polynucleotide encodes the mature region of SEQ ID NO:9.

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PRE                                PRO
1         11        21           31        41        51        61        71
VRSKKLWISL LFALALIFTM AFGSTSSAQA // AGKSNGEKKY IVGFKQTMST MSAAKKKDVI SEKGGKVQKQ FKYVDAASAT

                                MATURE FNA
81        91        101          108 111       121       131       141       151
LNEKAVKELK KDPSVAYVEE DHVAHAY // AQS  VPYGVSQIKA PALHSQGYTG SNVKVAVIDS GIDSSHPDLK VAGGASMVPS

161       171       181       191       201       211       221       231
ETNPFQDNNS HGTHVAGTVA ALNNSIGVLG VAPSASLYAV KVLGADGSGQ YSWIINGIEW AIANNMDVIN MSLGGPSGSA

241       251       261       271       281       291       301       311
ALKAAVDKAV ASGVVVVAAA GNEGTSGSSS TVGYPGKYPS VIAVGAVDSS NQRASFSSVG PELDVMAPGV SIQSTLPGNK

321       331       341       351       361       371       381
YGALNGTSMA SPHVAGAAAL ILSKHPNWTN TQVRSSLENT TTKLGDSFYY GKGLINVQAA AQ (SEQ ID NO:1)
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FIG. 1

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                                  1        10        20        30        40
FNA_B_Amyloliquefaciens           --VRSKKLWISLLFALALIFTMAFG-STSSAQAAGKSNGEKKYIVGFKQT
BPN_P00782_B_amyloliquefaciens    --MRGKKVWISLLFALALIFTMAFG-STSSAQAAGKSNGEKKYIVGFKQT
ACA34903_B_amyloliquefaciens      --MRGKKVWISLLFALALIFTMAFG-STTSAQAAGKSNGEKKYIVGFKQT
AAZ66858_B_amyloliquefaciens      --MRGKKVWISLLFALALIFTMAFG-STSPAQAAGKSNGEKKYIVGFKQT
AAT45900_Bacillus_sp_DJ4          --MRGKKVWISLLFALALIFTMAFG-STSPAQAAGKSNGEKKYIVGFKQT
YP_001420645_B_amyloliquefaci*    --MRGKKVWISLLFALALIFTMAFG-STSPAQAAGKSNGEKKYIVGFKQT
ABY25856_G_stearothermophilus     --MRGKKVWISLLFALALIFTMAFG-STSPAQAAGKSNGEKKYIVGFKQT
ABI93801_Bacillus_sp_RH219        --MRGKKVWISLLFALALIFTMAFG-STTSAQAAGKSNGEKKYIVGFKQT
AAV30845_Bacillus_sp_B16          --MRGKKVWISLLFALALIFTMAFG-STSPAQAAGKSNGEKKYIVGFKQT
ABY83469_Bacillus_subtilis        --MRGKKVWISLLFALALIFTMAFG-STSPAHAAGKSNGEKKYIVGFKQT
AAC63365_Bacillus_subtilis        --MRGKKVWISLLFALALIFTMAFG-STSPAQAAGKSNGEKKYIVGFKQT
ABJ99977_Bacillus_subtilis        --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
P35835_B_subtilis_subsp_natto     --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
P00783_B_subtilis_subsp_amyl*     --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
CAD62180_Bacillus_subtilis        --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
ABJ98766_Bacillus_subtilis        ----------MSLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
ABJ98765_Bacillus_subtilis        --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
AAX35771_Bacillus_subtilis        --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
AAO65246_Bacillus_subtilis        --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
P29142_G_stearothermophilus       --MRSKKLWISLLFALTLIFTMAFS-NMS-VQAAGKSSTEKKYIVGFKQT
CAB12870_B_subtilis_subsp_su*     --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
ABJ99976_Bacillus_subtilis        --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
AAX35772_Bacillus_subtilis        --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
1408206A_Bacillus_subtilis        --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
ZP_03590715_B_subtilis_subsp*     --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
ACL37472_Bacillus_subtilis        --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
AprE_NP_388911_B_subtilis         --MRSKKLWISLLFALTLIFTMAFS-NMS-VQAAGKSSTEKKYIVGFKQT
ACE63521_Bacillus_sp_ZLW2         --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
ABY65903_Bacillus_subtilis        --MRSKKLWISLLFALTLIFTMAFN-NMS-AQAAGKSSTEKKYIVGFKQT
AAX53176_Bacillus_subtilis        --MRSKKLWISLLFAFTLIFTMAFS-NMS-AQAAGKNSEEKKYIVGFKQT
ACJ07037_Bacillus_subtilis        --MRGKKVWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
CAE18180_Bacillus_subtilis        --MRSKKLWISLLFALTLIFTMAFS-NMS-AQAAGKSSTEKKYIVGFKQT
BAE92942_B_licheniformis          --MKKKSLWLSVLTALLLVLSTVFS-SPASAAQPAK-DVEKDYIVGFKSS
AAU88064_Bacillus_pumilus         MCVKKKNVMTSVLLAVPLLFSAGFGGTMANAETVSKTDSEKSYIVGFKAS
ABU68339_B_licheniformis          -MMRKKSFWLGMLTALMLVFTMAFS-DSASAAQPAK-NVEKDYIVGFKSG
ACM47735_Bacillus_pumilus         MCVKKKNVMTSVLLAVPLLFSAGFGGTMANAETVSKTDSEKSYIVGFKAS
CAJ70731_B_licheniformis          -MMRKKSFWLGMLTALMLVFTMAFS-DSASAAQPAK-NVEKDYIVGFKSG
AAT75303_Bacillus_mojavensis      -MMRKKSFWLGMLTAFMLVFTMAFG-DSASAAQPAK-NVEKDYIVGFKSG
YP_001486216_B_pumilus_SAFR0*     --MKKKNVMTSVLLAVPLLFSAGFGGSMANAETVSKSDSEKSYIVGFKAS
BAE79641_Bacillus_pumilus         MCVKKKNVMTSVLLAVPLLFSAGFGGSMANAETASKSESEKSYIVGFKAS
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FIG. 2A

FIG. 2B

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                                1       10        20        30        40
BAA93474_Bacillus_pumilus       MCVKKKNVMTSVLLAVPLLFSAGFGGSMANAETASKSESEKSYIVGFKAS
AAR19220_Bacillus_pumilus       MCVKKKNVMTSVLLAVPLLFSAGFGGSIANAETASKSESEKSYIVGFKAS
AAG31027_B_licheniformis        -MRKKSFWLGMLTALMLVFTMAFS-DSASAAQPAK-NVEKDYIVGFKSG
YP_078307_B_licheniformis       -MRKKSFWLGMLTALMLVFTMAFS-DSASAAQPAK-NVEKDYIVGFKSG
Carlsberg_P00780_B_licheniform  -MRKKSFWLGMLTAFMLVFTMAFS-DSASAAQPAK-NVEKDYIVGFKSG
ACM07731_Bacillus_pumilus       MCVKEKNVMTSVLLAVPLLFSAGFGGSMANAETVSKTDSEKSYIVGFKAS
ACA97991_B_licheniformis        -MRKKSFWLGMLTAFMLVFTMAFS-DSASAAQPAK-NVEKDYIVGFKSG
AAX14553_B_intermedius          --MKKKNVMTSVLLAVPLLFSAGFGGSMANAETVSKSASEKSYIVGFKAS
AAS86761_B_licheniformis        -MRKKSFWLGMLTAFMLVFTMAFS-DSASAAQPAK-NVEKDYIVGFKSG
AAG31028_B_licheniformis        -MRKKSFWLGMLTAFMLVFTMAFS-DSASAAQPAK-NVEKNYIVGFKSG
AAG31026_B_licheniformis        -MRKKSFWLGMLTAFMLVFTMAFS-DSASAAQPAK-NVEKDYIVGFKSG
AAY82467_B_licheniformis        -MRKKSFWLGMLTAFMLVFTMAFS-DSASAAQPAK-NVEKDYIVGFKSG
AAB34259_B_licheniformis        -MRKKSFWLGMLTAFMLVFTMAFS-DSASAAQPAK-NVEKDYIVGFKSG
CA003040_Bacillus_pumilus       MCVKKKNVMTSVLLAVPLLFSAGFGGSMANAETVSKTDSEKSYIVGFKAS
CAA62668_B_licheniformis        -MRKKSFWFGMLTAFMLVFTMEFS-DSASAAQPGK-NVEKDYFVGFKSG
CAA62667_B_licheniformis        -MRKKSFWLGMLTALMLVFTMAFS-DSASAAQPGK-NVEKDYIVGFKSG
CAA62666_B_licheniformis        -MRKKSFWLGMLTAFMLVFTMAFS-DSASAAQPAK-NVEKDYIVGFKSG
                                      .:* *. *::: *.  : .  .*  **.*:****
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		Percent(%) identity	SEQ ID NO:
FNA_B_Amyloliquefaciens	MSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAHAY	100.0	7
BPN_P00782_B_amyloliquefaciens	MSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAHAY	97.2	11
ACA34903_B_amyloliquefaciens	MSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAQAY	95.3	12
AAZ66858_B_amyloliquefaciens	MSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAQAY	95.3	13
AAT45900_Bacillus_sp_DJ4	MSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAQAY	95.3	14
YP_001420645_B_amyloliquefaci*	MSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAKAY	95.3	15
ABY25856_G_stearothermophilus	MSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAQAY	95.3	16
ABI93801_Bacillus_sp_RH219	MSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAQAY	95.3	17
AAV30845_Bacillus_sp_B16	MSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAQAY	95.3	18
ABY83469_Bacillus_subtilis	MSTMSAAKKKDVIFEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAQAY	93.5	19
AAC63365_Bacillus_subtilis	MSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAQAY	95.3	20
ABJ99977_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	21
P35835_B_subtilis_subsp_natto	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	22
P00783_B_subtilis_subsp_amyl*	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	23
CAD62180_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDAKAVKELKQDPSVAYVEEDHIAHQY	85.8	24
ABJ98766_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	86.9	25
ABJ98765_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	26
AAX35771_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	27
AAO65246_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	28

FIG. 2C

FIG. 2C-1

	50 60 70 80 90 100	Percent(%) identity	SEQ ID NO:
P29142_G_stearothermophilus	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	86.8	29
CAB12870_B_subtilis_subsp_su*	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	30
ABJ99976_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	31
AAX35772_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	32
1408206A_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	33
ZP_03590715_B_subtilis_subsp*	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	34
ACL37472_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKIQKQFKYVNAATATLNEKAVKELKQDPSVAYVEEDHIAHEY	86.8	35
AprE_NP_388911_B_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	86.8	36
ACE63521_Bacillus_sp_ZLW2	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	87.7	37
ABY65903_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEY	86.8	38
AAX53176_Bacillus_subtilis	MGAMSTAKKKDVISEKGGKVQKQFKYVNAAAATLDDKAVKELKKDPSVAYVEEDHVAHEY	84.9	39
ACJ07037_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAATATLDEKAVKELKQDPSVAYVEEDHIAHEY	84.9	40
CAE18180_Bacillus_subtilis	MSAMSSAKKKDVISEKGGKVQKQFKYVNAAAATLDEKAVKELKQDPSVAYVEEDHIALEY	85.8	41
BAE92942_B_licheniformis	VKTAAV--KKDVIKENGGKVDKQFKIINAAKATLDQEEVKALKKDPSVAYVEEDHIAHAM	59.6	42
AAU88064_Bacillus_pumilus	ATTNSS--KKQAVIQNGGKLEKQYRLINAAQVKMSEQAAKKLEHDPSIAYVEEDHKAEAY	46.7	43
ABU68339_B_licheniformis	VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHAL	56.7	44
ACM47735_Bacillus_pumilus	ATTNSS--KKQAVIQNGGKLEKQYRLINAAQVKMSEQAAKKLEHDPSIAYVEEDHKAEAY	46.7	45
CAJ70731_B_licheniformis	VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHAL	56.7	46
AAT75303_Bacillus_mojavensis	VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHAL	56.7	47
YP_001486216_B_pumilus_SAFR0*	ATTNSS--KKQAVTQNGGKLEKQYRLINAAQVKMSEQAAKKLEHDPSIAYVEEDHKAEAY	45.7	48
BAE79641_Bacillus_pumilus	ATTNSS--KKQAVTQNGGKLEKQYRLINAAQVKMSEQAAKKLEHDPSIAYVEEDHKAEAY	47.6	49
BAA93474_Bacillus_pumilus	ATTNSS--KKQAVTQNGGKLEKQYRLINAAQVKMSEQAAKKLEHDPSIAYVEEDHKAEAY	47.6	50
AAR19220_Bacillus_pumilus	ATTNSS--KKQAVTQNGGKLEKQYRLINAAQVKMYEQAAKKLEHDPSIAYVEEDHKAEAY	47.6	51
AAG31027_B_licheniformis	VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHAL	56.7	52

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YP_078307_B_licheniformis       VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALEEVKNDPDVAYVEEDHVAHAL  55.8    53
Carlsberg_P00780_B_licheniform  VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHAL  55.8    54
ACM07731_Bacillus_pumilus       ATTNSS--KKQAVIQNGGKLEKQYRLINAAQVKMSEQAAKKLEHDPSIAYVEEDHKAEAY  47.6    55
ACA97991_B_licheniformis        VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHAL  55.8    56
AAX14553_B_intermedius          ATTNSS--KKQAVTQNGGKLEKQYRLINAAQVKMSEQAAKKLEHDPSIAYVEEDHKAEAY  45.7    57
AAS86761_B_licheniformis        VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHAL  55.8    58
AAG31028_B_licheniformis        VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHAL  55.8    59
AAG31026_B_licheniformis        VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHAL  55.8    60
AAY82467_B_licheniformis        VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHAL  55.8    61
AAB34259_B_licheniformis        VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVAHAL  55.8    62
CA003040_Bacillus_pumilus       ATTNSS--KKQAVIQNGGKLEKQYRLINAAQVKMSEQAAKKLEHDPSIAYVEEDHKAEAY  47.6    63
CAA62668_B_licheniformis        VKTASV--KKDIIKESGGKVDKQFRIINAAKATLDKEALKEVKNDPDVAYVEEDHVAHAL  55.8    64
CAA62667_B_licheniformis        VKTASV--KKDIIKESGGKVDKQFRIINAGKAKLDKEALKEVKNDPDVAYVEEDHVAHVL  55.8    65
CAA62666_B_licheniformis        VKTASV--KKDIIKESGGKVDKQFRIINAAKAKLDKEALKEVKNDPDVAYVEEDHVGHGL  53.8    66
                                  : :   **: : :.***:.**:: ::*. ..:  :  : :::**.:** ***** .
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FIG. 2C-2

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                                 1        10        20        30        40        50
FNA_B_Amyloliquefaciens          AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASM
BPN_P00782_B_amyloliquefaciens   AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASM
ACA34903_B_amyloliquefaciens     AQSVPYGVSQIKAPALHSQGFTGSNVKVAVIDSGIDSSHPDLKVAGGASM
AAZ66858_B_amyloliquefaciens     AQSVPYGVSQIKAPALHSQGFTGSNVKVAVIDSGIDSSHPDLKVAGGASM
AAT45900_Bacillus_sp_DJ4         AQSVPYGVSQIKAPALHSQGFTGSNVKVAVIDSGIDSSHPDLKVAGGASM
YP_001420645_B_amyloliquefaci*   AQSVPYGVSQIKAPALHSQGFTGSNVKVAVIDSGIDSSHPDLKVAGGASM
ABY25856_G_stearothermophilus    AQSVPYGVSQIKAPALHSQGFTGSNVKVAVIDSGIDSSHPDLKVAGGASM
ABI93801_Bacillus_sp_RH219       AQSVPYGVSQIKAPALHSQGFTGSNVKVAVIDSGIDSSHPDLKVAGGASM
AAV30845_Bacillus_sp_B16         AQSVPYGVSQIKAPALHSQGFTGSNVKVAVIDSGIDSSHPDLKVAGGASM
ABY83469_Bacillus_subtilis       AQSVPYGVSQIKAPALHSQGFTGSNVKVAVIDSGIDSSHPDLKVAGGASM
AAC63365_Bacillus_subtilis       AQSVPYGVSQIKAPALHSQGFTGSNVKVAVIDSGIDSSHPDLKVAGGASM
ABJ99977_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
P35835_B_subtilis_subsp_natto    AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
P00783_B_subtilis_subsp_amyl*    AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
CAD62180_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
ABJ98766_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
ABJ98765_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
AAX35771_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
AAO65246_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
P29142_G_stearothermophilus      AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
CAB12870_B_subtilis_subsp_su*    AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
ABJ99976_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
AAX35772_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
1408206A_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
ZP_03590715_B_subtilis_subsp*    AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
ACL37472_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
AprE_NP_388911_B_subtilis        AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
ACE63521_Bacillus_sp_ZLW2        AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
ABY65903_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPHLNVRGGASF
AAX53176_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
ACJ07037_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVKGGASF
CAE18180_Bacillus_subtilis       AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASF
BAE92942_B_licheniformis         AQTVPYGIPLIKADKVQAQGYKGANVKVGIIDTGIASSHTDLKVVGGASF
AAU88064_Bacillus_pumilus        AQTVPYGIPQIKAPAVHAQGYKGANVKVAVLDTGIHAAHPDLNVAGGASF
ABU68339_B_licheniformis         AQTVPYGVPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
ACM47735_Bacillus_pumilus        AQTVPYGIPQIKAPAVHAQGYKGANVKVAVLDTGIHAAHPDLNVAGGASF
CAJ70731_B_licheniformis         AQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
AAT75303_Bacillus_mojavensis     AQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
YP_001486216_B_pumilus_SAFR0*    AQTVPYGIPQIKAPAVHAQGYKGANVKVAVLDTGIHAAHPDLNVAGGASF
BAE79641_Bacillus_pumilus        AQTVPYGIPQIKAPAVHAQGYKGANVKVAVLDTGIHAAHPDLNVAGGASF
```

FIG. 3A

```
                                1        10        20        30        40        50
BAA93474_Bacillus_pumilus       AQTVPYGIPQIKAPAVHAQGYKGANVKVAVLDTGIHAAHPDLNVAGGASF
AAR19220_Bacillus_pumilus       AQTVPYGIPQIKAPAVHAQGYKGANVKVAVLDTGIHAAHPDLNVAGGASF
AAG31027_B_licheniformis        AQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
YP_078307_B_licheniformis       AQTVPYGIPLIKADKVQAQGYKGANVKVAVLDTGIQASHPDLNVVGGASF
Carlsberg_P00780_B_licheniform  AQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
ACM07731_Bacillus_pumilus       AQTVPYGIPQIKAPAVHAQGYKGANVKVAVLDTGIHAAHPDLNAAGGASF
ACA97991_B_licheniformis        AQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
AAX14553_B_intermedius          AQTVPYGIPQIKAPAVHAQGYKGANVKVAVLDTGIHAAHPDLNVAGGASF
AAS86761_B_licheniformis        AQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
AAG31028_B_licheniformis        AQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
AAG31026_B_licheniformis        AQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
AAY82467_B_licheniformis        AQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
AAB34259_B_licheniformis        AQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
CAO03040_Bacillus_pumilus       AQTVPYGIPQIKAPAVHAQGYKGANVKVAVLDTGIHAAHPDLNVAGGASF
CAA62668_B_licheniformis        GQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
CAA62667_B_licheniformis        GQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
CAA62666_B_licheniformis        GQTVPYGIPLIKADKVQAQGFKGANVKVAVLDTGIQASHPDLNVVGGASF
                                .*:****:. ***  :::**:.*:****.::*:** ::*..*:. ****:

                                51        60        70        80        90        100
FNA_B_Amyloliquefaciens         VPSETNPFQDNNSHGTHVAGTVAALNNSIGVLGVAPSASLYAVKVLGADG
BPN_P00782_B_amyloliquefaciens  VPSETNPFQDNNSHGTHVAGTVAALNNSIGVLGVAPSASLYAVKVLGADG
ACA34903_B_amyloliquefaciens    VPSETNPFQDNNSHGTHVAGTVAALNNSVGVLGVAPSASLYAVKVLGADG
AAZ66858_B_amyloliquefaciens    VPSETNPFQDNNSHGTHVAGTVAALNNSVGVLGVAPSASLYAVKVLGADG
AAT45900_Bacillus_sp_DJ4        VPSETNPFQDNNSHGTHVAGTVAALNNSVGVLGVAPSASLYAVKVLGADG
YP_001420645_B_amyloliquefaci*  VPSETNPFQDYNSHGTHVAGTVAALNNSVGVLGVAPSASLYAVKVLGADG
ABY25856_G_stearothermophilus   VPSETNPFQDNNSHGTHVAGTVAALNNSVGVLGVAPSASLYAVKVLGADG
ABI93801_Bacillus_sp_RH219      VPSETNPFQDNNSHGTHVAGTVAALNNSVGVLGVAPSASLYAVKVLGADG
AAV30845_Bacillus_sp_B16        VPSETNPFQDNNSHGTHVAGTVAALNNSVRVLGGAPSASLYAVKVLGADG
ABY83469_Bacillus_subtilis      VPSETNPFQDNNSHGTHVAGTVAALNNSVGVLGVAPSASLYAVKVLGADG
AAC63365_Bacillus_subtilis      VPSETNPFQDNNSHGTHVAGTVAALNNSVFVLGVAPSASLYAVKVLGADG
ABJ99977_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTG
P35835_B_subtilis_subsp_natto   VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTG
P00783_B_subtilis_subsp_amyl*   VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVSPSASLYAVKVLDSTG
CAD62180_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTVAALNNSIGVLGVAPNASLYAVKVLDSTG
ABJ98766_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTG
ABJ98765_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTG
AAX35771_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTG
AAC65246_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTG
P29142_G_stearothermophilus     VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVSPSASLYAVKVLDSTG
CAB12870_B_subtilis_subsp_su*   VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTG
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FIG. 3B

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                                51        60        70        80        90        100
ABJ99976_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTG
AAX35772_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTG
1408206A_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGYIAALNNSIGVLGVSPSASLYAVKVLDSTG
ZP_03590715_B_subtilis_subsp*   VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVSPSASLYAVKVLDSTG
ACL37472_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPNASLYAVKVLDSTG
AprE_NP_388911_B_subtilis       VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVSPSASLYAVKVLDSTG
ACE63521_Bacillus_sp_ZLW2       VPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTG
ABY65903_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTIAALNNSICVLGVAPSASLYAVKVLDSTG
AAX53176_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTVAALNNTIGVLGVAPSASLYAVKVLDSTG
ACJ07037_Bacillus_subtilis      VPSETNPYQDGSSHGTHVAGTIAALNNTIGVLGVAPNASLYAVKVLDSTG
CAE18180_Bacillus_subtilis      VPSETNPYQGRSSHGTHVAGTISAFNNSIGVLGVAPNASLYAVKVLDSTG
BAE92942_B_licheniformis        VSGESYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPNVSLYAIKVLNSSG
AAU88064_Bacillus_pumilus       VPSEPNATQDFQSHGTHVAGTIAALDNTIGVLGVAPNASLYAVKVLDRNG
ABU68339_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
ACM47735_Bacillus_pumilus       VPSEPNATQDFQSHGTHVAGTIAALDNTIGVLGVAPNASLYAVKVLDRNG
CAJ70731_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
AAT75303_Bacillus_mojavensis    VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
YP_001486216_B_pumilus_SAFR0*   VPSEPNATQDFQSHGTHVAGTIAALDNTIGVLGVAPSASLYAVKVLDRYG
BAE79641_Bacillus_pumilus       VPSEPNATQDFQSHGTHVAGTIAALDNTIGVLGVAPSASLYAVKVLDRYG
BAA93474_Bacillus_pumilus       VPSEPNATQDFQSHGTHVAGTIAALDNTIGVLGVAPSASLYAVKVLDRYG
AAR19220_Bacillus_pumilus       VPSEPNATQDFQSHGTHVAGTIAALDNTIGVLGVAPSASLYAVKVLDRYG
AAG31027_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
YP_078307_B_licheniformis       VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPNVSLYAVKVLNSSG
Carlsberg_P00780_B_licheniform  VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
ACM07731_Bacillus_pumilus       VPSEPNATQDFQSHGTHVAGTIAALDNTIGVLGVAPSASLYAVKALDRNG
ACA97991_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
AAX14553_B_intermedius          VPSEPNATQDFQSHGTHVAGTIAALDNTIGVLGVAPSASLYAVKVLDRNG
AAS86761_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
AAG31028_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
AAG31026_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
AAY82467_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
AAB34259_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
CAO03040_Bacillus_pumilus       VPSEPNATQDFQSHGTHVAGTIAALDNTIGVLGVAPSASLYAVKVLDRNG
CAA62668_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLFAVKVLNSSG
CAA62667_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
CAA62666_B_licheniformis        VAGEAYNT-DGNGHGTHVAGTVAALDNTTGVLGVAPSVSLYAVKVLNSSG
                                *..*.    . ..******* ::*::*:  *** :*..**:*:*.*.  *

                                101       110       120       130       140       150
FNA_B_Amyloliquefaciens         SGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGVVVV
BPN_P00782_B_amyloliquefaciens  SGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGVVVV
```

FIG. 3C

```
                                101       110       120       130       140       150
ACA34903_B_amyloliquefaciens    SGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGVVVV
AAZ66858_B_amyloliquefaciens    SGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGIVVV
AAT45900_Bacillus_sp_DJ4        SGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGVVVV
YP_001420645_B_amyloliquefaci*  SGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGVVVV
ABY25856_G_stearothermophilus   SGQYSWIINGIEWAIAYNMDVINMSLGGPSGSAALKAAVDKAVASGIVVV
ABI93801_Bacillus_sp_RH219      SGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGVVVV
AAV30845_Bacillus_sp_B16        SGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGIVVV
ABY83469_Bacillus_subtilis      SGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGIVVV
AAC63365_Bacillus_subtilis      SGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGVVVV
ABJ99977_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPSGSTALKTVVDKAVSSGIVVA
P35835_B_subtilis_subsp_natto   SGQYSWIINGIEWAISNNMDVINMSLGGPTGSTALKTVVDKAVSSGIVVA
P00783_B_subtilis_subsp_amyl*   SGQYSWIINGIEWAISNNMDVINMSLGGPSGSTALKTVVDKAVSSGIVVA
CAD62180_Bacillus_subtilis      NGQYSWIINGIEWAISNKMDVINMSLGGPSGSTALKSVVDRAVASGIVVV
ABJ98766_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPSGSTALKTVVDKAVSSGIVVA
ABJ98765_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPSGSTALKTVVDKAASSGIVVA
AAX35771_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPTGSTALKTVVDKAVSSGIVVA
AAO65246_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPTGSTALKTVVDKAVSSGIVVA
P29142_G_stearothermophilus     SGQYSWIINGIEWAISNNMDVINMSLGGPSGSTALKTVVDKAVSSGIVVA
CAB12870_B_subtilis_subsp_su*   SGQYSWIINGIEWAISNNMDVINMSLGGPTGSTALKTVVDKAVSSGIVVA
ABJ99976_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPTGSTALKTVVDKAVSSGIVVA
AAX35772_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPTGSTALKTVVDKAVSSGIVVA
1408206A_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPSGSTALKTVVDKAVSSGIVVA
ZP_03590715_B_subtilis_subsp*   SGQYSWIINGIEWAISNNMDVINMSLGGPTGSTALKTVVDKAVSSGIVVA
ACL37472_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPSGSTALKTVVDKAVSNGIVVA
AprE_NP_388911_B_subtilis       SGQYSWIINGIEWAISNNMDVINMSLGGPTGSTALKTVVDKAVSSGIVVA
ACE63521_Bacillus_sp_ZLW2       SGQYSWIINGIEWAISNNMDIINMSLGGPTGSTALKTVVDKAVSSGIVVA
ABY65903_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPTGSTALKTVVDKAVSSGIVVA
AAX53176_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPTGSTALKTVVDKAVASGIVVV
ACJ07037_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPSGSTALKTVVDKAVSSGIVVA
CAE18180_Bacillus_subtilis      SGQYSWIINGIEWAISNNMDVINMSLGGPSGSTALKTVVDKAVSSGIVVA
BAE92942_B_licheniformis        SGTYSAIVSGIEWATQNGLDVINMSLGGPSGSTALKQAVDKAYASGIVVV
AAU88064_Bacillus_pumilus       DGQYSWIISGIEWAVANNMDVINMSLGGPSGSTALKNAVDTANNRGVVVV
ABU68339_B_licheniformis        SGSYSGIVSGIEWATTNGMDVINMSLGGASGSTAMKQAVDNAYARGVVVV
ACM47735_Bacillus_pumilus       DGQYSWIISGIEWAVANNMDVINMSLGGPSGSTALKNAVDTANNRGVVVV
CAJ70731_B_licheniformis        SGSYSGIVSGIEWATTNGMDVINMSLGGASGSTAMKQAVDNAYARGVVVV
AAT75303_Bacillus_mojavensis    SGSYSGIVSGIEWATTNGMDVINMSLGGASGSTAMKQAVDNAYARGVVVV
YP_001486216_B_pumilus_SAFR0*   DGQYSWIISGIEWAVANNMDVINMSLGGPNGSTALKNAVDTANNRGVVVV
BAE79641_Bacillus_pumilus       DGQYSWIISGIEWAVANNMDVINMSLGGPNGSTALKNAVDTANNRGVVVV
BAA93474_Bacillus_pumilus       DGQYSWIISGIEWAVANNMDVINMSLGGPNGSTALKKAVDTANNRGVVVV
AAR19220_Bacillus_pumilus       DGQYSWIISGIEWAVANNMDVINMSLGGPNGSTALKNAVDTANNRGVVVV
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FIG. 3D

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                                 101       110       120       130       140       150
AAG31027_B_licheniformis         SGSYSGIVSGIEWATTNGMDVINMSLGGASGSTAMKQAVDNAYARGVVVV
YP_078307_B_licheniformis        SGSYSGIVSGIEWATTNGMDVINMSLGGPSGSTAMKQAVDNAYARGVVVV
Carlsberg_P00780_B_licheniform   SGTYSGIVSGIEWATTNGMDVINMSLGGPSGSTAMKQAVDNAYARGVVVV
ACM07731_Bacillus_pumilus        DGQYSWIISGIEWAVANNMDVINMSLGGASGSTALKNAVDTANSRGVVAV
ACA97991_B_licheniformis         SGSYSGIVSGIEWATTNGMDVINMSLGGASGSTAMKQAVDNAYAKGVVVV
AAX14553_B_intermedius           DGQYSWIISGIEWAVANNMDVINMSLGGPNGSTALKNAVDTANNRGVVVV
AAS86761_B_licheniformis         SGSYSGIVSGIEWATTNGMDVINMSLGGASGSTAMKQAVDNAYARGVVVV
AAG31028_B_licheniformis         SGSYSGIVSGIEWATTNGMDVINMSLGGASGSTAMKQAVDNAYARGVVVV
AAG31026_B_licheniformis         SGSYSGIVSGIEWATTNGMDVINMSLGGASGSTAMKQAVDNAYAKGVVVV
AAY82467_B_licheniformis         SGSYSGIVSGIEWATTNGMDVINMSLGGASGSTAMKQAVDNAYARGVVVV
AAB34259_B_licheniformis         SGSYSGIVSGIEWATTNGMDVINMSLGGASGSTAMKQAVDNAYARGVVVV
CAO03040_Bacillus_pumilus        DGQYSWIISGIEWAVANNMDVINMSLGGPNGSTALKNAVDTANNRGVVVV
CAA62668_B_licheniformis         SGSYSGIVSGIEWATTNGMDVINMSLGGPSGSTAMKQAVDNAYSKGVVPV
CAA62667_B_licheniformis         SGSYSAIVSGIEWATTTGMDVINMSLGGASVSTAMKQAVDHAYARGAVVV
CAA62666_B_licheniformis         SGSYSGIVSGIEWVTTNGMDVINMSLGGASGSTAMKQAVDNAYARGVVVV
                                 .* ** *:.****.    :*:******** *:*:* .** *   * * .

                                 151       160       170       180       190       200
FNA_B_Amyloliquefaciens          AAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGPELDVMA
BPN_P00782_B_amyloliquefaciens   AAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGPELDVMA
ACA34903_B_amyloliquefaciens     AAAGNEGTSGGSSTVGYPGKYPSVIAVGAVNSSNQRASFSSVGSELDVMA
AAZ66858_B_amyloliquefaciens     AAAGNEGTSGSSSTVGYPGKYPSVIAVGAVNSSNQRASFSSVGSELDVMA
AAT45900_Bacillus_sp_DJ4         AAAGNEGTSGGSSTVGYPGKYPSVIAVGAVNSSNQRASFSSVGSELDVMA
YP_001420645_B_amyloliquefaci*   AAAGNEGTSGGSSTVGYPGKYPSVIAVGAVNSSNQRASFSSVGSELDVMA
ABY25856_G_stearothermophilus    AAAGNEGTSGSSSTVGYPGKYPSVIAVGAVNSSNQRASFSSVGSELDVMA
ABI93801_Bacillus_sp_RH219       AAAGNEGTSGGSSTVGYPGKYPSVIAVGAVNSSNQRASFSSVGSELDVMA
AAV30845_Bacillus_sp_B16         AAAGNEGTSGSSSTVGYPGKYPSVIAVGAVNSSNQRASFSSVGSELDVMA
ABY83469_Bacillus_subtilis       AAAGNEGTSGGSSTVGYPGKYPSVIAVGAVNSSNQRASFSSVGSELDVMA
AAC63365_Bacillus_subtilis       AAAGNEGTSGGSSTVGYPGKYPSVIAVGAVNSSNQRASFSSVGSELDVMA
ABJ99977_Bacillus_subtilis       AAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
P35835_B_subtilis_subsp_natto    AAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSVGSELDVMA
P00783_B_subtilis_subsp_amyl*    AAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
CAD62180_Bacillus_subtilis       AAAGNEGTSGSSSTIGYPAKYPSTIAVGAVNSSNQRGSFSSVGPELDVMA
ABJ98766_Bacillus_subtilis       AAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
ABJ98765_Bacillus_subtilis       AAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
AAX35771_Bacillus_subtilis       AAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
AAO65246_Bacillus_subtilis       AAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSVGSELDVMA
P29142_G_stearothermophilus      AAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
CAB12870_B_subtilis_subsp_su*    AAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
ABJ99976_Bacillus_subtilis       AAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
AAX35772_Bacillus_subtilis       AAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSEFDVMA
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FIG. 3E

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                                151       160       170       180       190       200
1408206A Bacillus subtilis      AAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
ZP_03590715_B_subtilis_subsp*   AAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
ACL37472_Bacillus_subtilis      AAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGPELDVMA
AprE_NP_388911_B_subtilis       AAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
ACE63521_Bacillus_sp_ZLW2       AAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
ABY65903_Bacillus_subtilis      AAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
AAX53176_Bacillus_subtilis      AAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
ACJ07037_Bacillus_subtilis      AAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGSELDVMA
CAE18180_Bacillus_subtilis      AAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSTQRASFSSAGSELDVMA
BAE92942_B_licheniformis        AAAGNSGSSGSQNTIGYPAKYDSVIAVGAVDSNKNRASFSSVGSELEVMA
AAU88064_Bacillus_pumilus       AAAGNSGSSGSRSTVGYPAKYDSTIAVANVNSSNVRNSSSSAGPELDVSA
ABU68339_B_licheniformis        AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
ACM47735_Bacillus_pumilus       AAAGNSGSSGSRSTVGYPAKYDSTIAVANVNSNNVRNSSSSAGPELDVSA
CAJ70731_B_licheniformis        AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
AAT75303_Bacillus_mojavensis    AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
YP_001486216_B_pumilus_SAFR0*   AAAGNSGSTGSTSTVGYPAKYDSTIAVANVNSSNVRNSSSSAGPELDVSA
BAE79641_Bacillus_pumilus       AAAGNSGSTGSTSTVGYPAKYDSTIAVANVNSNNVRNSSSSAGPELDVSA
BAA93474_Bacillus_pumilus       AAAGNSGSTGSTSTVGYPAKYDSTIAVANVNSNNVRNSSSSAGPELDVSA
AAR19220_Bacillus_pumilus       AAAGNSGSTGSTSTVGYPAKYDSTIAVANVNSNNVRNSSSSAGPELDVSA
AAG31027_B_licheniformis        AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
YP_078307_B_licheniformis       AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
Carlsberg_P00780_B_licheniform  AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
ACM07731_Bacillus_pumilus       AAAGNSGSSGSRSTVGYPAKYESTIAVANVNSNNVRNSSSSAGPELDVSA
ACA97991_B_licheniformis        AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
AAX14553_B_intermedius          AAAGNSGSTGSTSTVGYPAKYDSTIAVANVNSSNVRNSSSSAGPELDVSA
AAS86761_B_licheniformis        AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
AAG31028_B_licheniformis        AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
AAG31026_B_licheniformis        AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
AAY82467_B_licheniformis        AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
AAB34259_B_licheniformis        AAAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
CAO03040_Bacillus_pumilus       AAAGNSGSFGSTSTVGYPAKYDSTIAVANVNGNNVRNSSSSAGPELDVSA
CAA62668_B_licheniformis        AAAGNSGSSGYTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
CAA62667_B_licheniformis        SSAGNSGSSGNTNTIGYPAKYDSVIAVGAVDSNSNRASFSSVGAELEVMA
CAA62666_B_licheniformis        AAAGNSGSSGNTNTIGYPAKCDSVIPVGGEDSNSNRSSFSSVGAELEVMA
                                ::***.*: *  .*:***.*  *.*.*.  :... * * **.*.*::* *

                                201       210       220       230       240       250
FNA_B_Amyloliquefaciens         PGVSIQSTLPGNKYGALNGTSMASPHVAGAAALILSKHPNWTNTQVRSSL
BPN_P00782_B_amyloliquefaciens  PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSL
ACA34903_B_amyloliquefaciens    PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSL
AAZ66858_B_amyloliquefaciens    PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSL
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FIG. 3F

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                                201       210       220       230       240       250
AAT45900_Bacillus_sp_DJ4        PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSL
YP_001420645_B_amyloliquefaci*  PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSL
ABY25856_G_stearothermophilus   PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSL
ABI93801_Bacillus_sp_RH219      PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSL
AAV30845_Bacillus_sp_B16        PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSL
ABY83469_Bacillus_subtilis      PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSL
AAC63365_Bacillus_subtilis      PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILFKHPNWTNTQVRSSL
ABJ99977_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
P35835_B_subtilis_subsp_natto   PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
P00783_B_subtilis_subsp_amyl*   PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
CAD62180_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
ABJ98766_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
ABJ98765_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
AAX35771_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
AAO65246_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
P29142_G_stearothermophilus     PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
CAB12870_B_subtilis_subsp_su*   PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
ABJ99976_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
AAX35772_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
1408206A_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
ZP_03590715_B_subtilis_subsp*   PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
ACL37472_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
AprE_NP_388911_B_subtilis       PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
ACE63521_Bacillus_sp_ZLW2       PGVSIQSTLPGGTYGAYNGTSMATPHVAGATALILSKHPTWTNAQVRDRL
ABY65903_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
AAX53176_Bacillus_subtilis      PGVSIQSTLPGGTYGSYNGTSMATPHVAGAAALILSKHPTWSNAQVRDRL
ACJ07037_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
CAE18180_Bacillus_subtilis      PGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRL
BAE92942_B_licheniformis        PGVSVYSTYPSNTYTSLNGTSMASPHVAGAAALILSKYPTLSASQVRNRL
AAU88064_Bacillus_pumilus       PGTSILSTVPSSGYTSYTGTSMASPHVAGAAALILSKNPNLTNSQVRQRL
ABU68339_B_licheniformis        PGAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
ACM47735_Bacillus_pumilus       PGTSILSTVPSSGYTSYTGTSMASPHVAGAAALILSKNPNLTNSQVRQRL
CAJ70731_B_licheniformis        PGAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
AAT75303_Bacillus_mojavensis    PGAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
YP_001486216_B_pumilus_SAFR0*   PGTSILSTVPSSGYTSYTGTSMASPHVAGAAALILSKYPNLSTTQVRQRL
BAE79641_Bacillus_pumilus       PGTSILSTVPSSGYTSYTGTSMASPHVAGAAALILSKYPNLSTSQVRQRL
BAA93474_Bacillus_pumilus       PGTSILSTVPSSGYTSYTGTSMASPHVAGAAALILSKYPNLSTSQVRQRL
AAR19220_Bacillus_pumilus       PGTSILSTVPSSGYTSYTGTSMASPHVAGAAALILSKYPNLSTSQVRQRL
AAG31027_B_licheniformis        PGAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
YP_078307_B_licheniformis       PGAGVYSTYPTSTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
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FIG. 3G

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                          201       210       220       230       240       250
Carlsberg_P00780_B_licheniform  PGAGVYSTYPTSTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
ACM07731_Bacillus_pumilus       PGTSILSTVPSSGYTSYTGTSMASPHVAGAAALILSKNPNLTNSQVRQRL
ACA97991_B_licheniformis        PGAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
AAX14553_B_intermedius          PGTSILSTVPSSGYTSYTGTSMASPHVAGAAALILSKNPNLSNSQVRQRL
AAS86761_B_licheniformis        PGAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
AAG31028_B_licheniformis        PGAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
AAG31026_B_licheniformis        PGAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
AAY82467_B_licheniformis        PGAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
AAB34259_B_licheniformis        PGAGVYSTYPTNTYATLNGTSMVSPHVAGAAALILSKHPNLSASQVRNRL
CA003040_Bacillus_pumilus       PGTSILSTVPSSGYTSYTGTSMASPHVAGAAALILSKYPNLSTSQVRQRL
CAA62668_B_licheniformis        PGAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRNRL
CAA62667_B_licheniformis        PGAGVYSTYPTNTYATLNGTSMASPHVAGAAALILSKHPNLSASQVRTRL
CAA62666_B_licheniformis        PVSGVYSTYPTNTYTTLNGTSMASPHVAGTSALILSKHPNLSASQVRNRL
                                *  .: ** * . * : .****.:*****:;**** * *. : :***  *

                          251       260       270          Percent(%) ID    SEQ ID NO:
FNA_B_Amyloliquefaciens         ENTTTKLGDSFYYGKGLINVQAAAQ        100.0            9
BPN_P00782_B_amyloliquefaciens  ENTTTKLGDSFYYGKGLINVQAAAQ         99.6            67
ACA34903_B_amyloliquefaciens    ENTTTKLGDAFYYGKGLINVQAAAQ         97.5            68
AAZ66858_B_amyloliquefaciens    ENTTTKLGDAFYYGKGLINVQAAAQ         97.5            69
AAT45900_Bacillus_sp_DJ4        ENTTTKLGDAFYYGKGLINVQAAAQ         97.5            70
YP_001420645_B_amyloliquefaci*  ENTTTKLGDAFYYGKGLINVQAAAQ         97.1            71
ABY25856_G_stearothermophilus   ENTTTKLGDAFYYGKGLINVQAAAQ         97.1            72
ABI93801_Bacillus_sp_RH219      ENTATKLGDAFYYGKGLINVQAAAQ         97.1            73
AAV30845_Bacillus_sp_B16        ENTTTKLGDAFYYGKGLINVQAAAQ         96.7            74
ABY83469_Bacillus_subtilis      ENTTTKLGDAFYYGKGLINVQAAAQ         97.1            75
AAC63365_Bacillus_subtilis      ENTTTKLGDAFYYGKGLINVQAAAH         96.4            76
ABJ99977_Bacillus_subtilis      ESTATYLGNSFYYGKGLINVQAAAQ         86.9            77
P35835_B_subtilis_subsp_natto   ESTATYLGNSFYYGKGLINVQAAAQ         86.5            78
P00783_B_subtilis_subsp_amyl*   ESTATYLGNSFYYGKGLINVQAAAQ         86.5            79
CAD62180_Bacillus_subtilis      ESTTTYLGNSFYYGKGLINVQAAAQ         87.3            80
ABJ98766_Bacillus_subtilis      ESTATYLGNSFYYGKGLINVQAAAQ         86.9            81
ABJ98765_Bacillus_subtilis      ESTATYLGNSFYYGKGLINVQAAAQ         86.5            82
AAX35771_Bacillus_subtilis      ESTATYLGNSFYYGKGLINVQAAAQ         86.5            83
AAO65246_Bacillus_subtilis      ESTATYLGSSFYYGKGLINVQAAAQ         86.5            84
P29142_G_stearothermophilus     ESTATYLGNSFYYGKGLINVQAAAQ         86.5            85
CAB12870_B_subtilis_subsp_su*   ESTATYLGNSFYYGKGLINVQAAAQ         86.2            86
ABJ99976_Bacillus_subtilis      ESTATYLGSSFYYGKGLINVQAAAQ         86.2            87
AAX35772_Bacillus_subtilis      ESTATYLGNSFYYGKGLINVQAAAQ         86.2            88
1408206A_Bacillus_subtilis      ESTATYLGNSFYYGKGLINVQAAAQ         86.2            89
```

FIG. 3H

	251 260 270	Percent(%) ID	SEQ ID NO:
ZP_03590715_B_subtilis_subsp*	ESTATYLGNSFYYGKGLINVQAAAQ	85.8	90
ACL37472_Bacillus_subtilis	ESTATYLGNSFYYGKGLINVQAAAQ	86.2	91
AprE_NP_388911_B_subtilis	ESTATYLGNSFYYGKGLINVQAAAQ	85.8	92
ACE63521_Bacillus_sp_ZLW2	ESTATYLGSSFYYGKGLINVQAAAQ	85.5	93
ABY65903_Bacillus_subtilis	ESTATYLGNSFYYGKGLINVQAAAQ	85.8	94
AAX53176_Bacillus_subtilis	ESTATNLGSSFYYGKGLINVQAAAQ	86.2	95
ACJ07037_Bacillus_subtilis	ESTATYLGSSFYYGKGLINVQAAAQ	85.8	96
CAE18180_Bacillus_subtilis	ESTATYLGNSFYYGKGLINVQAAAQ	84.7	97
BAE92942_B_licheniformis	SSTATNLGDSFYYGKGLINVEAAAQ	71.2	98
AAU88064_Bacillus_pumilus	ENTATPLGDSFYYGKGLINVQAASN	75.3	99
ABU68339_B_licheniformis	SSTATYLGSSFYYGKGLINVEAAAQ	70.4	100
ACM47735_Bacillus_pumilus	ENTATPLGDSFYYGKGLINVQAASN	74.9	101
CAJ70731_B_licheniformis	SSTATYLGSSFYYGKGLINVEAAAQ	70.1	102
AAT75303_Bacillus_mojavensis	SSTATYLGSSFYYGKGLINVEAAAQ	70.1	103
YP_001486216_B_pumilus_SAFR0*	ENTATPLGNSFYYGKGLINVQAASN	74.2	104
BAE79641_Bacillus_pumilus	ENTATPLGNSFYYGKGLINVQAASN	73.5	105
BAA93474_Bacillus_pumilus	ENTATPLGNSFYYGKGLINVQAASN	73.5	106
AAR19220_Bacillus_pumilus	ENTATPLGNSFYYGKGLINVQAASN	73.5	107
AAG31027_B_licheniformis	SSTATYLGSSFYYGKGLINV-----	69.9	108
YP_078307_B_licheniformis	SSTATYLGSSFYYGKGLINVEAAAQ	70.1	109
Carlsberg_P00780_B_licheniform	SSTATYLGSSFYYGKGLINVEAAAQ	70.1	110
ACM07731_Bacillus_pumilus	ENTATPLGDSFYYGKGLINVQAASN	73.8	111
ACA97991_B_licheniformis	SSTATYLGSSFYYGKGLINVEAAAQ	70.1	112
AAX14553_B_intermedius	ENTATPLGNSFYYGKGLINAQAASN	73.8	113
AAS86761_B_licheniformis	SSTATYLGSSFYYGKGLINVEAAAQ	70.1	114
AAG31028_B_licheniformis	SSTATYLGSSFYYGKGLINV-----	69.9	115
AAG31026_B_licheniformis	SSTATYLGSSFYYGKGLINV-----	69.9	116
AAY82467_B_licheniformis	SSTATYLGSSFYYGKGLINVEGAAQ	69.7	117
AAB34259_B_licheniformis	SSTATYLGSSFYYGKGLINVEAAAQ	69.7	118
CAO03040_Bacillus_pumilus	ENTATPLGDSFYYGKGLINVQAASN	73.5	119
CAA62668_B_licheniformis	SSTATYLGSSFYYGKGLINVEAAAQ	69.0	120
CAA62667_B_licheniformis	SRTATYLGSSFSYGRGLINVEAAAQ	67.2	121
CAA62666_B_licheniformis	SRTATYLGSSFYYGKGLINVEAAAQ	66.4	122
	. *:* **.:* **:****.		

FIG. 3I

FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4

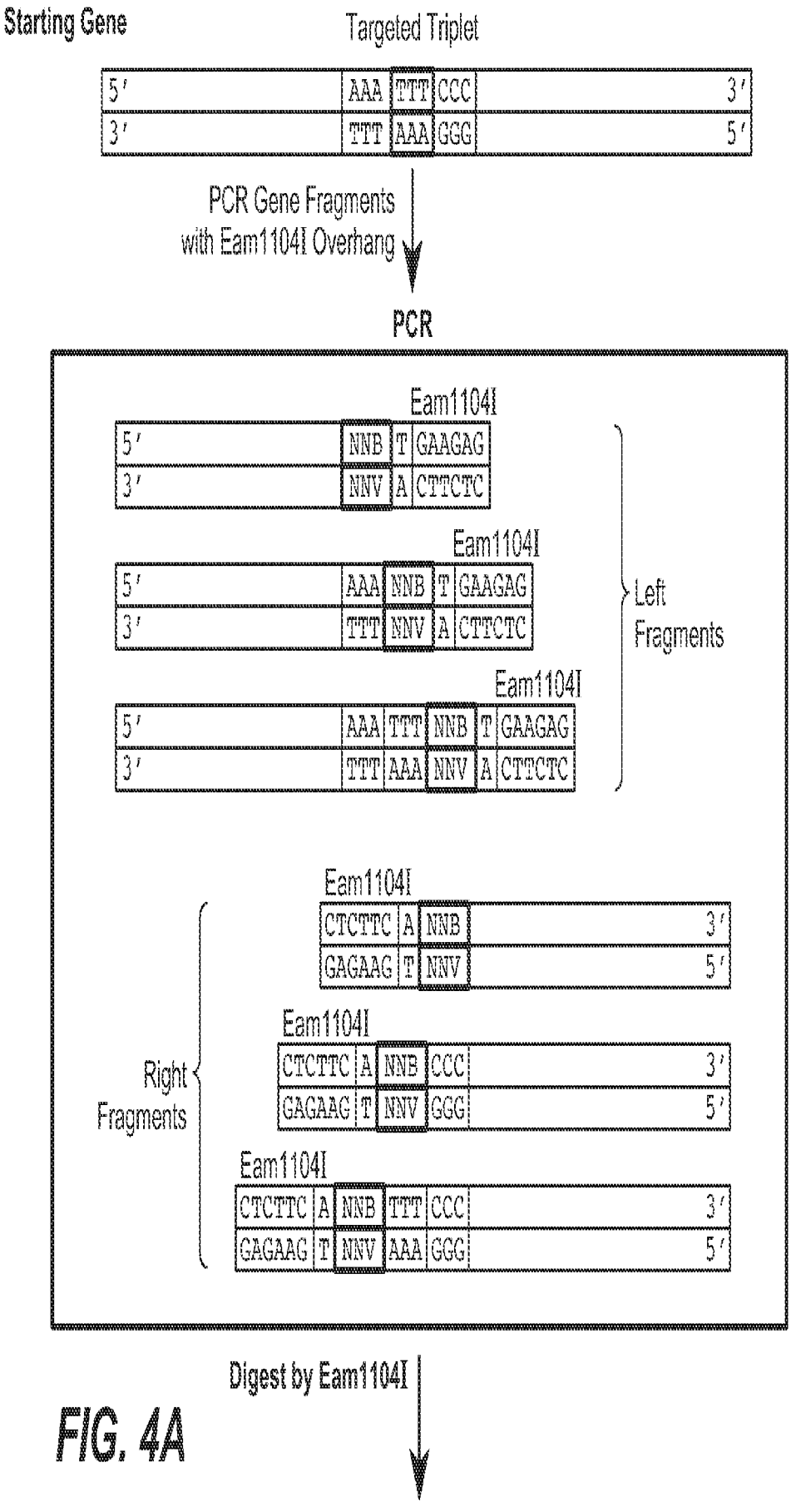
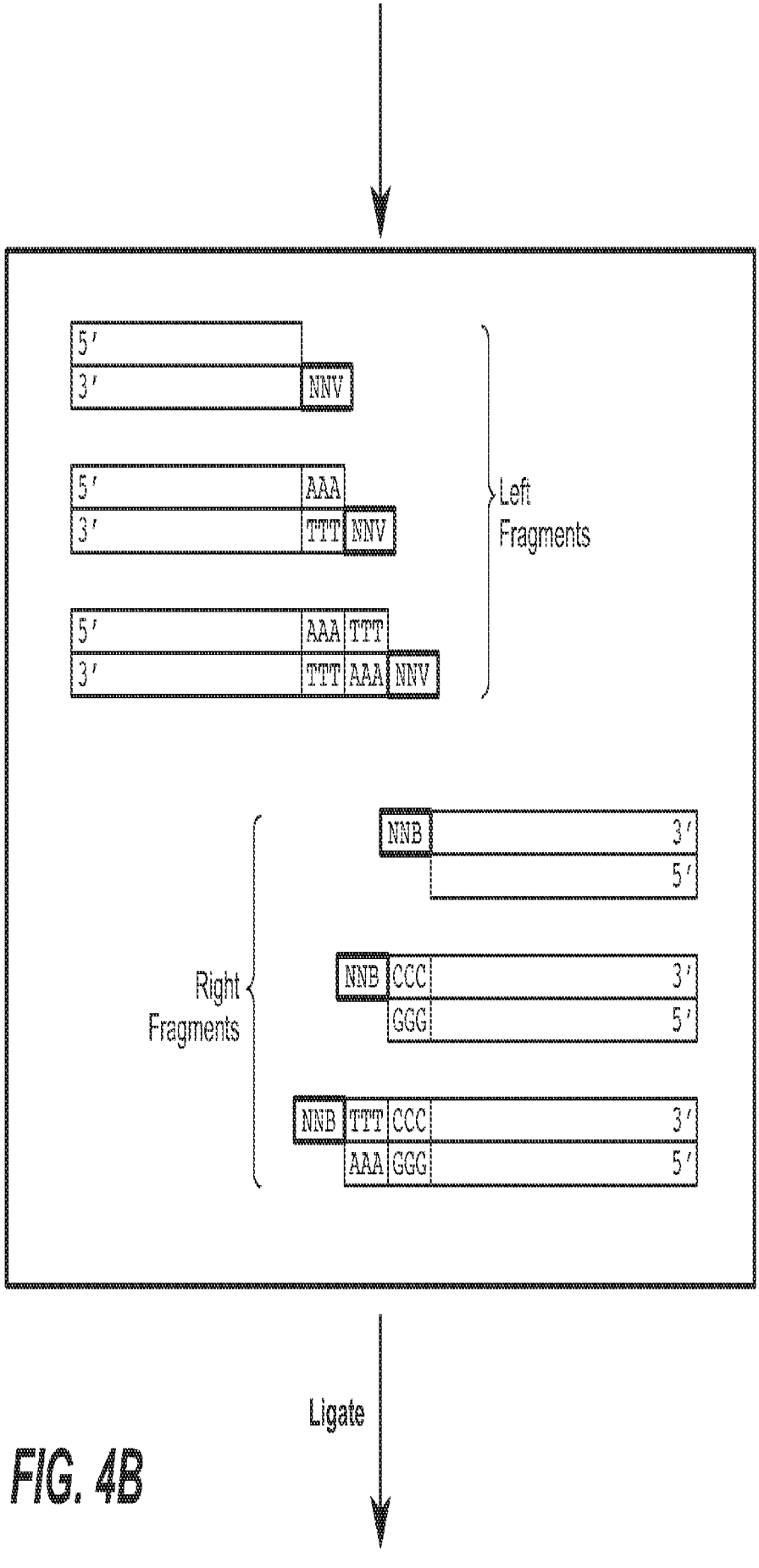


FIG. 4A


5′
3′
NNV
5′
AAA
3′
TTT
NNV
5′
AAA
TTT
3′
TTT
AAA
NNV
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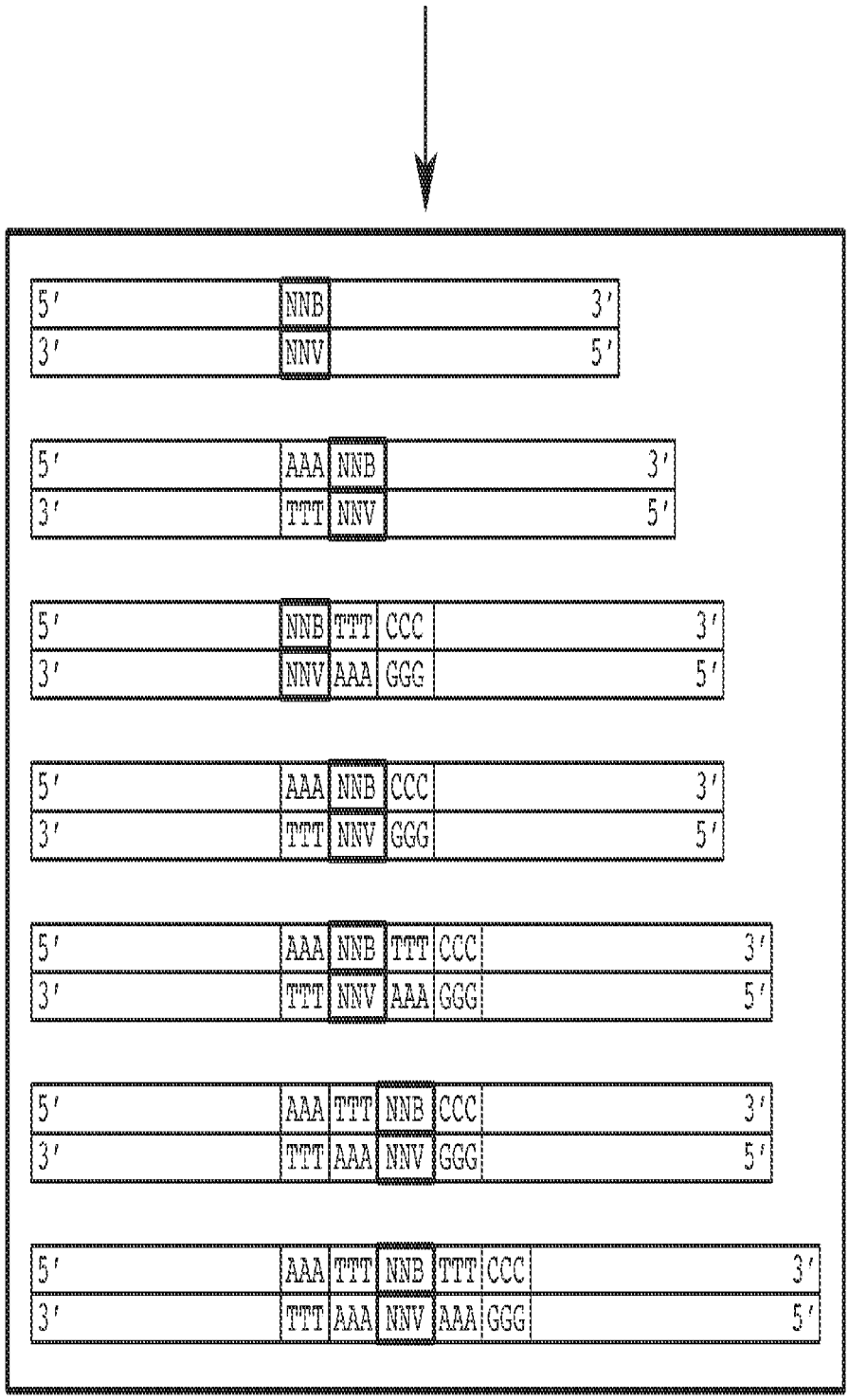
Fragments
NNB
3′
5′
Right
Fragments
NNB
CCC
3′
GGG
5′
NNB
TTT
CCC
3′
AAA
GGG
5′
Ligate

FIG. 4B



Examples of the Resulting Mutants

FIG. 4C

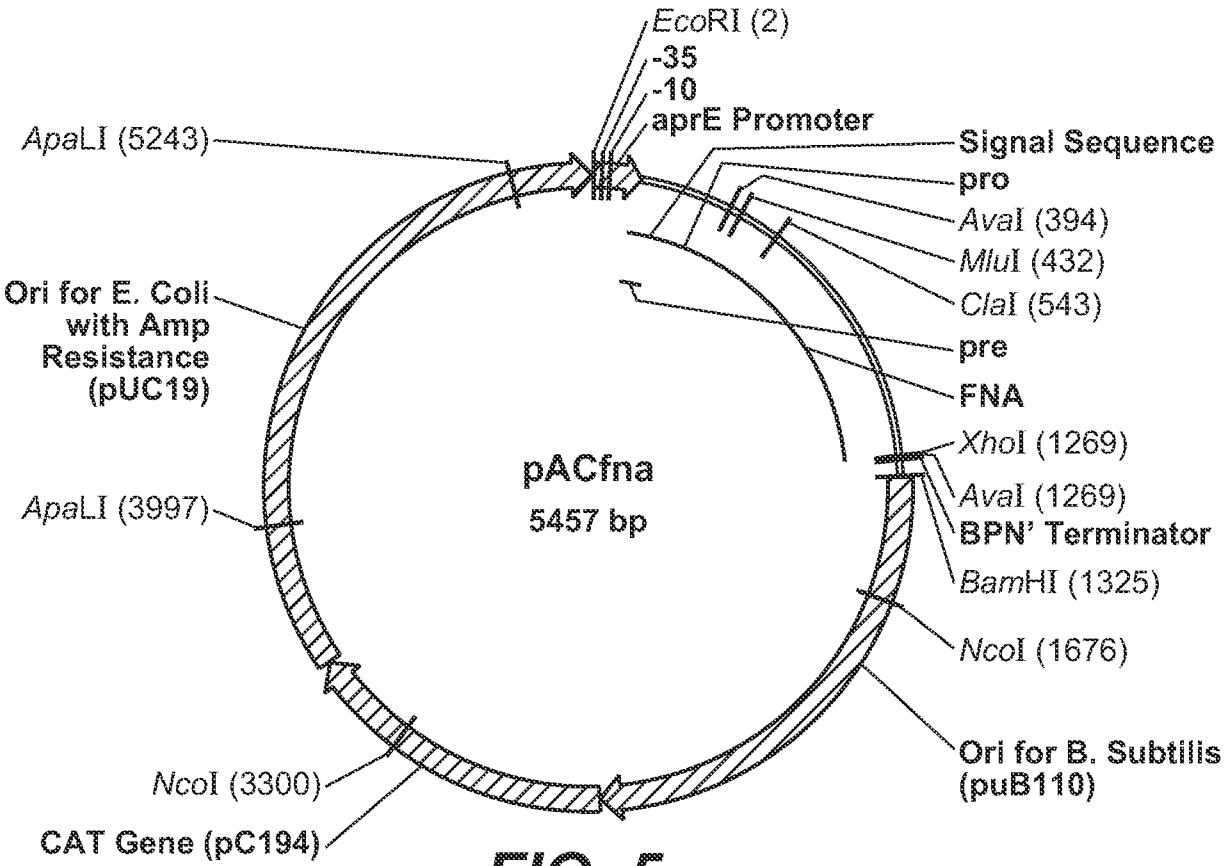

EcoRI (2)
-35
-10
aprE Promoter
Signal Sequence
pro
AvaI (394)
MluI (432)
ClaI (543)
pre
FNA
XhoI (1269)
AvaI (1269)
BPN' Terminator
BamHI (1325)
NcoI (1676)
Ori for B. Subtilis (puB110)
ApaLI (5243)
Ori for E. Coli with Amp Resistance (pUC19)
ApaLI (3997)
NcoI (3300)
CAT Gene (pC194)
pACfna
5457 bp

FIG. 5

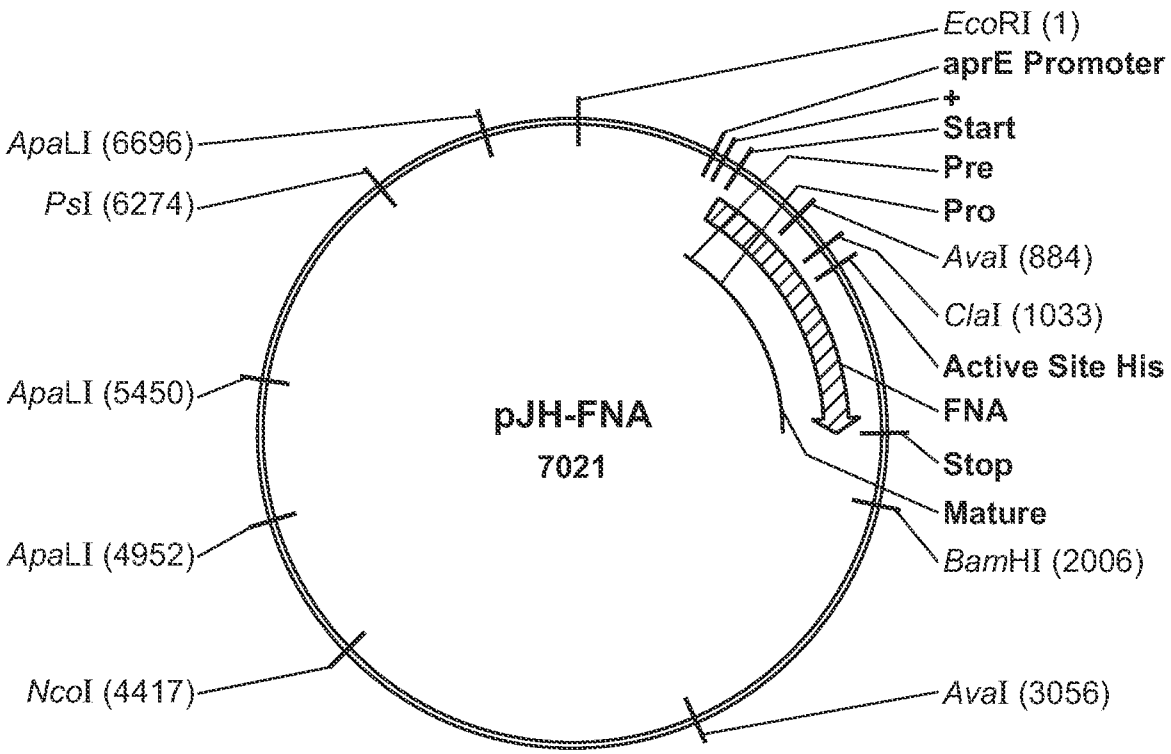

EcoRI (1)
aprE Promoter
+
Start
Pre
Pro
AvaI (884)
ClaI (1033)
Active Site His
FNA
Stop
Mature
BamHI (2006)
AvaI (3056)
ApaLI (6696)
PsI (6274)
ApaLI (5450)
ApaLI (4952)
NcoI (4417)
pJH-FNA
7021

FIG. 6

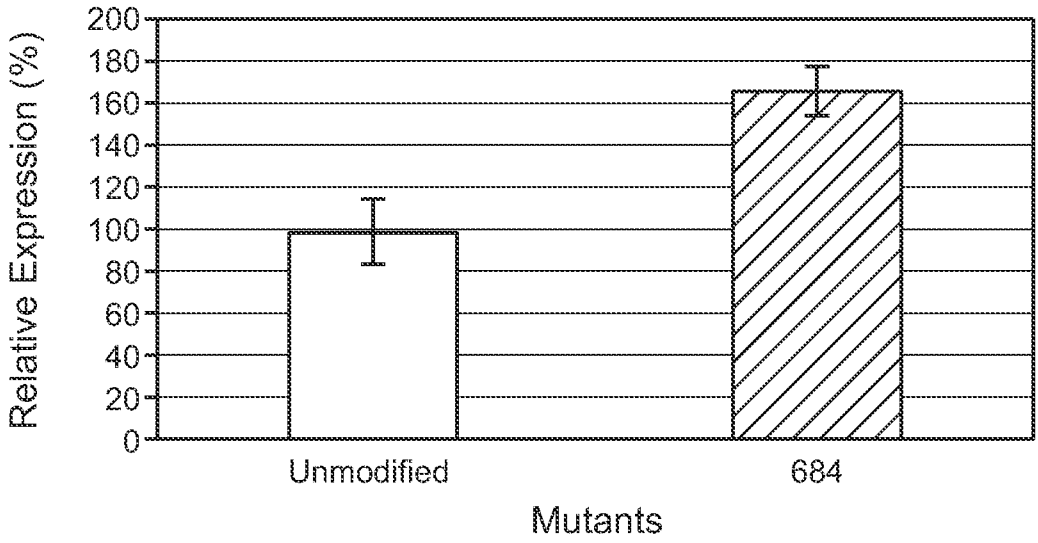

Relative Expression (%)
200
180
160
140
120
100
80
60
40
20
0
Unmodified
684
Mutants

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/031283

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/54 C12N15/57 C07H21/04
ADD.

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)
C12N C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/072321 A1 (SATO TSUYOSHI [JP] ET AL) 15 April 2004 (2004-04-15) the whole document	1-36

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"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

"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

"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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 August 2010	17/08/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Scheffzyk, Irmgard

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2010/031283

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2004072321 A1	15-04-2004	CN 1487080 A	07-04-2004
		DE 10328887 A1	29-04-2004
		DK 200300925 A	27-12-2003
		US 2006105428 A1	18-05-2006