Engineering Signal Peptide for Enhanced Protein Secretion in *Lactococcus lactis*

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Supplementary Methods

Mathematical model

We developed a mathematical model to quantitatively explain the differences in secretion due to silent mutations in the signal peptide. The model relates the stability of mRNA secondary structure around the ribosome binding site to translation and secretion efficiencies based on a previous model with modifications (1). Nomenclature and parameter values are provided in Table S1.

First, we modeled translation as a process initiated by a ribosome recognizing the ribosome binding site and unfolding of the initiation region in the mRNA (2, 3). The folding/unfolding equilibrium of mRNA is based specifically on the free energy of mRNA secondary structure around the ribosome binding site (RBS) (ΔG_{RBS}).

$$U \xleftarrow{K_f} \rightarrow F$$

where $K_f = [F]/[U]$ and $\Delta G_{RBS} = - RT \ln(K_f)$ (S1)

The 30S subunit of the ribosome binds to the unfolded mRNA with association constant K_{rb}.

$$Rb + U \xleftarrow{Krb} Rb{\bullet}U$$

where $K_{rb} = [Rb{\bullet}U]/[Rb][U]$ and $\Delta G_{rb} = - RT \ln(K_{rb})$ (S2)

Since initiation is the rate-limiting step in translation (4), translation was set to be proportional to the amount of ribosome-bound mRNA ([Rb•U]). Finally, since *L. lactis* only secretes a fraction of the expressed protein (5), secretion rate was calculated as a fraction of the total amount of protein translated:

$$\text{secretion} \propto \text{protein expression} \propto [Rb{\bullet}U]$$

so, $\text{secretion} = C\,[Rb{\bullet}U]/Rb_{tot}$ (S3)

where C is a constant that is unique to the amino acid sequence of a specific signal peptide.

Together with the two mass balance equations:

$$[U] + [F] + [Rb{\bullet}U] = RNA_{tot} \quad \text{(S4)}$$

and $[Rb] + [Rb{\bullet}U] = Rb_{tot}$, (S5)

the system of equations, (S1), (S2), (S4) and (S5), was solved in Matlab using *fsolve* to obtain the unknowns [U], [F], [Rb], and [Rb•U]. The C values for the Usp45sp and Usp45TM8 silent mutation libraries were determined experimentally from the secretion levels of wild-type Usp45sp and Usp45TM8, respectively, and Equation S3 was used to calculate the final normalized protein secretion.

Supplementary Tables

Table S1. Nomenclature and parameter values used in the mathematical model.

Symbol	Description	Value	Reference
F	mRNA with secondary structure around the RBS (folded)	Calculated from *fsolve*	N/A
U	mRNA with exposed RBS (unfolded)	Calculated from *fsolve*	N/A
Rb	Free ribosomes	Calculated from *fsolve*	N/A
Rb•U	Ribosomes bound to unfolded mRNA	Calculated from *fsolve*	N/A
K_f	Equilibrium association constant for mRNA folding around the RBS	Calculated from ΔG_f	(6, 7)
ΔG_{RBS}	Free energy of mRNA secondary structure around the RBS	Varies from -8.5 to -0.5 kcal/mol (calculated from mfold with initiation term)	(6, 7)
K_{rb}	Equilibrium association constant for binding of 30S ribosomal subunit to unfolded mRNA	Calculated from ΔG_{rb}	(1)
ΔG_{rb}	Free energy of binding of 30S ribosomal subunit to unfolded mRNA	-14 kcal/mol	(1)
Rb_{tot}	Total concentration of ribosomes in the cell	40 µM (assumed to be equivalent to *E. coli* with the same doubling time)	(8, 9)
RNA_{tot}	Total concentration of mRNA transcripts (depending of induction level)	0.08 Rb_{tot}, 0.8 Rb_{tot}, and 8 Rb_{tot} for 0.1, 1, and 10 ng/ml nisin, respectively	This work
C	Peptide-specific proportionality constant relating protein expression to secretion efficiency	Usp45SM: 1.01; Usp45TM8_SM: 1.45	This work
R	Gas constant	1.986 cal/K mol	N/A
T	Temperature (in Kelvin)	303.15 K (*L. lactis* was grown at 30^{o}C)	N/A

Table S2. Bacterial strains and plasmids used in this study.

	Characteristics	Sources
Strains		
E. coli EC1000	RepA$^+$ MC1000, Km^r, carrying a single copy of the pWV01 *repA* gene in *glgB*	(10)
L. lactis NZ9000	*L. lactis* MG1353 (*nisRK* genes on the chromosome)	(11)
Plasmids		
pNZ8048m:usp45sp-SCI57his	Modified pNZ8048 containing P*nisA* promoter with Usp45sp fused with downsream RGS-His-tagged *SCI-57* gene; Cm^r	(9)
pNZ8048m:usp45sp-MazF	Modified pNZ8048 containing P*nisA* promoter with Usp45sp fused with downsream RGS-His-tagged *E. coli MazF* gene; Cm^r	This work
pNZ8048m:usp45sp-Nuc	Modified pNZ8048 containing P*nisA* promoter with Usp45sp fused with downsream RGS-His-tagged *S. aureus* nuclease gene; Cm^r	This work
pNZ8048m:usp45sp-AmyE	Modified pNZ8048 containing P*nisA* promoter with Usp45sp fused with downsream RGS-His-tagged *B. subtilis* α-amylase gene (with internal EcoRI silent mutation); Cm^r	This work
pNZ8048m:SP310-Nuc	Modified pNZ8048 containing P*nisA* promoter with SP310 signal peptide fused with downsream RGS-His-tagged *S. aureus* nuclease gene; Cm^r	This work
pNZ8048m:SP310-AmyE	Modified pNZ8048 containing P*nisA* promoter with SP310 signal peptide fused with downsream RGS-His-tagged *B. subtilis* α-amylase gene (with internal EcoRI silent mutation); Cm^r	This work

Table S3. Primers used in this study.

Primer Number	Primer Name	Nucleotide Sequence (5' → 3') [a]
1	MazF_BspEI_f	CTTACGTCCGGAGTTTACGCTATGGTAAGCCGATACGTAC
2	MazF_BamHI_r	TACGTTGGATCCTCTCCCAATCAGTACGTTAATTTTG
3	Usp45NcoIMazF-BspEI_f	TTCAGCTCCGGAGTTTACGCCATGGTAAGCC
4	SaurNuc_NcoI_f	TTTACGCCATGG**T**TTCACAAACAGATAATGGCGTAAATAG
5	SaurNuc_BamHI_r	ATGGTGGGATCCTCTTTGACCTGAATCAGCGTTGTCTTC
6	BsubAmy_NcoI_f	TTTACGCCATGGTTCTTACAGCACCGTCGATCAAAAG
7	BsubAmy_BamHI_r	ATGGTGGGATCCTCTATGGGGAAGAGAACCGCTTAAGC
8	BsubAmy_EcoRImut_f	CACGCAGAACTCATTGCTCG
9	BsubAmy_EcoRImut_r	CGAGCAATGAGTTCTGCGTG
10	SP310_EcoRI_f	CTCAAAGAATTCATGAAATTTAATAAAAAAAGAGTTGCAATAG
11	SP310_NcoI_r	TTGTCGCCATGGCATTAGTTTGGTTATCTTGGATTGATG
12	pNZ_EcoRI_f	TTATAAGGAGGCACTCAAAGAATTCATG
13	NucB_NcoI_r	CATTATCTGTTTGTGAAACCATGG
14	Usp45SM_NcoI_r	CACTTTCCATGGCRTANACNCCNGAYAANGGNGCNGCNGCNGAN AGDATNACNGTNGACATYAADATNGCNGADATDATYTTYTTYTT CATGAATTCTTTGAGTGCCTCCTTATAA
15	Usp45TM_NcoI_r	ATCGGCTTACCATGGCMNNAACMNNGGAMNNCGGGGCTGCMNNM NNAAGMNNCACMNNMNNMNNTAAMNNAGCMNNMNNMNNCTTTTT TTTCATGAATTCTTTGAGTGCCTCCTTATAA
16	TM8SM_NcoI_r [b]	GTGAAACCATGGCYTTNACRTTNGADATNGGNGCNGCNGTNGTN AGCATNACNACNACNGC**N**AARTGNGCYTTNAGNACYTTYTTYTT CATGAATTCTTTGAGTGCCTCCTTATAA

[a] Restriction sites are underlined.

[b] The bolded N should be a Y to encode for only silent mutations of leucine. Nevertheless, all clones selected for characterization in this work contained only silent mutations at this position.

Table S4. Nucleotide sequences of selected clones from the Usp45sp silent mutation library.

Usp45sp silent mutation clone	Nucleotide sequence (5' → 3')
Usp45sp	ATGAAAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTGATACTTTCTGCTGC AGCCCCGTTGTCCGGAGTTTACGCC
Usp45SM2	ATGAAGAAAAAGATCATATCCGCCATCTTGATGTCAACAGTCATTCTATCGGCCGC CGCCCCCTTATCAGGCGTGTATGCC
Usp45SM3	ATGAAGAAGAAGATCATTTCTGCCATTTTGATGTCAACAGTTATTCTTTCCGCAGC CGCTCCGTTATCCGGAGTGTACGCC
Usp45SM4	ATGAAGAAGAAAATCATTTCAGCGATATTAATGTCCACGGTCATCCTTTCTGCGGC CGCTCCGTTGTCTGGTGTCTACGCC
Usp45SM5	ATGAAGAAGAAAATCATCTCAGCCATATTAATGTCCACAGTGATCCTCTCCGCGGC AGCGCCATTGTCCGGCGTGTACGCC
Usp45SM6	ATGAAGAAGAAGATCATCTCCGCCATATTAATGTCCACTGTGATACTGTCAGCAGC TGCGCCGTTGTCAGGTGTATATGCC
Usp45SM7	ATGAAAAAAAAAATCATTTCAGCCATCTTGATGTCCACAGTTATCCTCTCAGCAGC AGCTCCCTTATCGGGAGTTTACGCC
Usp45SM8	ATGAAGAAGAAGATCATATCCGCGATCTTGATGTCGACAGTAATACTTTCGGCCGC GGCGCCGTTATCGGGGGTATATGCC
Usp45SM9	ATGAAAAAGAAGATCATCTCTGCCATATTAATGTCCACCGTAATCCTCTCCGCCGC CGCGCCCTTATCCGGAGTCTACGCC
Usp45SM10	ATGAAAAAGAAGATCATCTCAGCCATTTTGATGTCTACGGTTATTCTTTCTGCAGC AGCTCCATTGTCCGGCGTATACGCC
Usp45SM11	ATGAAGAAGAAAATCATCTCCGCAATATTAATGTCCACCGTGATACTATCTGCAGC TGCCCCGTTATCTGGAGTGTACGCC
Usp45SM12	ATGAAGAAGAAAATAATTTCCGCGATCTTAATGTCAACAGTGATCCTATCGGCAGC GGCGCCATTATCAGGAGTGTACGCC
Usp45SM14	ATGAAAAAGAAGATCATATCCGCCATCTTGATGTCTACAGTAATCCTTTCCGCGGC CGCCCCCTTATCAGGTGTGTATGCC
Usp45SM15	ATGAAGAAAAAGATCATCTCCGCAATTTTGATGTCGACAGTCATCCTCTCGGCTGC AGCACCTTTGTCGGGAGTCTACGCC
Usp45SM16	ATGAAGAAGAAGATAATATCCGCCATCTTAATGTCGACCGTGATACTTTCGGCAGC TGCTCCTTTATCGGGAGTATATGCC
Usp45SM19	ATGAAAAAAAAAATCATCTCTGCCATCTTAATGTCAACGGTTATTCTCTCCGCCGC

	TGCGCCTTTATCAGGGGTTTATGCC
Usp45SM20	ATGAAAAAGAAGATCATTTCGGCGATATTGATGTCCACCGTCATTCTATCCGCGGC TGCACCTTTGTCCGGGGTATACGCC

Table S5. Nucleotide sequences of selected clones from the Usp45sp targeted mutation library.

Usp45sp targeted mutation clone	**Nucleotide sequence (5' → 3')**
Usp45sp	ATGAAAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTGATACTTTCTGCTGC AGCCCCGTTGTCCGGAGTTTACGCC
Usp45TM6	ATGAAAAAAAAGCTTGTTACTGCTCAGTTATATGGGAGTGTGATTCTTCTGTCTGC AGCCCCGTATTCCTGTGTTAATGCC
Usp45TM7	ATGAAAAAAAAGAGTTGTACGGCTGGTTTACAGTTGATGGTGACGCTTTTGACTGC AGCCCCGTTTTCCTATGTTAATGCC
Usp45TM8	ATGAAAAAAAAGGTGCTGAAGGCTCATTTAGCTGTGGTTGTGATGCTTACGACGGC AGCCCCGATTTCCAATGTTAAGGCC
Usp45TM11	ATGAAAAAAAAGCCGAGGCGGGCTTTGTTATGTATGCTTGTGGGTCTTGCTCTGGC AGCCCCGATGTCCAATGTTACGGCC
Usp45TM12	ATGAAAAAAAAGCGGCGGATGGCTTGGTTACGTATGATGGTGGCTCTTGGGGTTGC AGCCCCGCCTTCCGATGTTCTTGCC
Usp45TM13	ATGAAAAAAAAGCGTGCGTCTGCTTTGTTAAAGCTGACTGTGCTGCTTACTACGGC AGCCCCGTGGTCCGTGGTTGGGGCC
Usp45TM14	ATGAAAAAAAAGTCGGCGCGTGCTGCTTTAATGCTTTCTGTGAATCTTTTTTTGGC AGCCCCGGCGTCCAATGTTAATGCC
Usp45TM28	ATGAAAAAAAAGCCGTCGAGTGCTGTGTTATTGTGTTGTGTGATGCTTGGTATTGC AGCCCCGTGTTCCTTTGTTATGGCC
Usp45TM29	ATGAAAAAAAAGCCGTGTACTGCTCTTTTACTTTGGGGTGTGGCTCTTGCGATTGC AGCCCCGAAGTCCTTTGTTCATGCC

Table S6. Nucleotide sequences of selected clones from the Usp45TM8 silent mutation library.

Usp45TM8 silent mutation clone	**Nucleotide sequence (5' → 3')**
Usp45TM8	ATGAAAAAAAAGGTGCTGAAGGCTCATTTAGCTGTGGTTGTGATGCTTACGACGG CAGCCCCGATTTCCAATGTTAAGGCC
Usp45TM8_SM7	ATGAAAAAAAAGGTCCTTAAAGCACACTTGGCCGTGGTAGTGATGCTAACGACTG CTGCCCCGATTTCTAATGTAAAAGCC
Usp45TM8_SM9	ATGAAAAAGAAGGTGCTCAAAGCACATTTAGCTGTGGTGGTGATGCTTACAACTG CTGCTCCCATTTCTAACGTTAAAGCC
Usp45TM8_SM10	ATGAAAAAAAAGGTCCTCAAGGCTCACTTGGCAGTGGTGGTAATGCTTACCACAG CGGCCCCTATCTCTAATGTCAAGGCC
Usp45TM8_SM14	ATGAAAAAGAAGGTCCTCAAGGCCCACTTGGCCGTAGTCGTTATGCTAACCACGG CTGCTCCAATATCTAACGTAAAAGCC
Usp45TM8_SM15	ATGAAAAAGAAGGTACTCAAAGCACATTTAGCTGTTGTCGTAATGCTCACAACGG CCGCTCCTATATCGAATGTCAAAGCC
Usp45TM8_SM22	ATGAAAAAGAAGGTCCTTAAGGCACATTTAGCTGTAGTCGTTATGCTAACTACGG CAGCCCCAATCTCAAATGTGAAAGCC
Usp45TM8_SM25	ATGAAAAAGAAGGTCCTCAAGGCCCACTTAGCAGTTGTTGTGATGCTCACCACAG CTGCCCCTATCTCGAATGTTAAAGCC

Supplementary Figures

Figure S1. Standard curve of α-amylase starch azure test. *B. subtilis* α-amylase (100 µl of 0.0001 mg/ml to 0.1 mg/ml solution) was mixed with 20 mg starch azure, and then 900 µl α-amylase buffer was added. Samples were shaken horizontally at 37°C for 1 hr, and absorbance of the supernatant was measured at 595 nm. The data represent the mean ± SEM of two independent experiments.

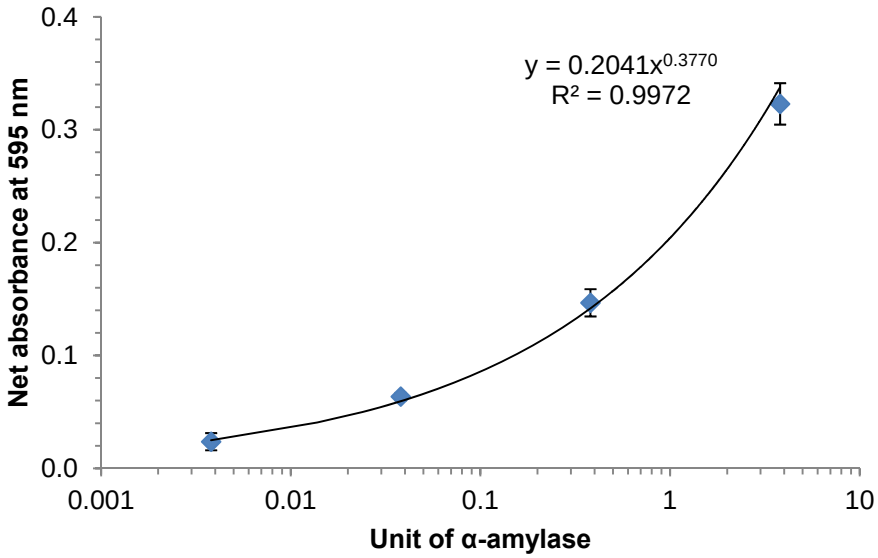
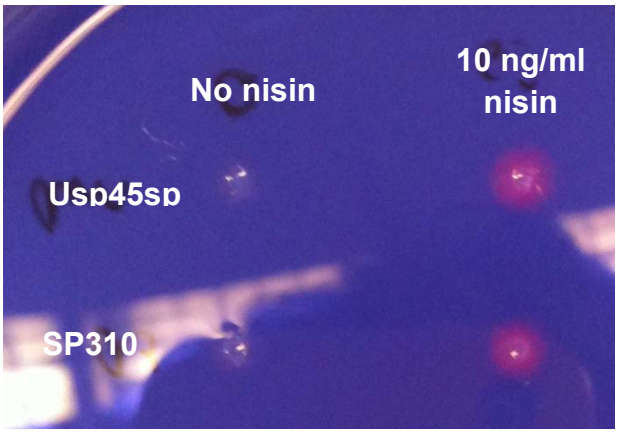
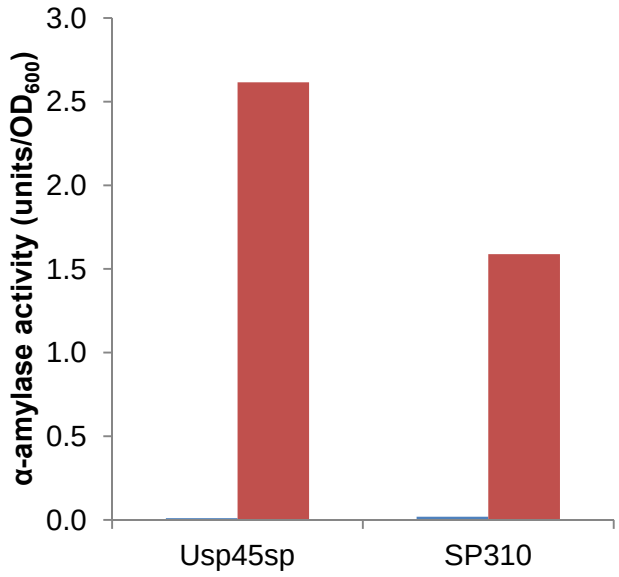


Figure S2. Nuclease plate test and α-amylase starch azure test of the two control signal peptides, Usp45sp and SP310. (A) The size of the pink halo on Toluidine blue-DNA-agar corresponds to the activity of secreted nuclease. (B) The starch azure test gives a more quantitative measure of α-amylase secretion (see Fig. S1). Blue bar: no nisin induction; red bar: 10 ng/ml nisin induction. In both cases, the secretion efficiency of Usp45sp is higher than that of SP310. In the starch azure test, SP310 secretion was ~62% of Usp45sp secretion, in agreement with previous results (12).

A



B



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