Elucidating the Role and Structure-Activity Relationships of the *Streptococcus oligofermentans* Competence Stimulating Peptide

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Supporting Information

Full Experimental Methods

General: Unless otherwise specified, all peptide synthesis reactions were manually conducted in 6 mL polypropylene reaction vessels containing porous frits for ease of filtering and washing the resin between reaction steps. All references to equivalents are used relative to the resin loading.

Chemical Reagents and Instrumentation: All chemical reagents and solvents were purchased from Aapptec, Sigma-Aldrich, or Chem-Impex and used without further purification. 18 MΩ water was purified using a Barnstead Smart2Pure Pro water purification system. Solid-Phase resin was purchased from Advanced ChemTech and Chem-Impex. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed using a Shimadzu system equipped with a CBM-20A communications bus module, two LC-20AT pumps, an SIL-20A autosampler, an SPD-20A UV/VIS detector, a CTO-20A column oven, and an FRC-10A fraction collector. All RP-HPLC solvents (18 MΩ water and HPLC-grade acetonitrile (ACN)) contained 0.1% trifluoroacetic acid (TFA). Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) data were obtained on a Bruker Microflex spectrometer equipped with a 60 Hz nitrogen laser and a reflectron. In positive ion mode, the acceleration voltage on Ion Source 1 was 19.01 kV. Exact mass (EM) data were obtained on an Agilent Technologies 6230 TOF LC/MS spectrometer. The samples were sprayed with a capillary voltage of 3500 V and the electrospray ionization (ESI) source parameters were as follows: gas temperature of 325 °C at a drying gas flow rate of 8 L/min at a pressure of 35 psi. MS/MS analysis of the extracted CSP was conducted at the Mick Hitchcock Nevada Proteomics Center at UNR using a Thermo-Fisher Scientific Eclipse Tribrid mass spectrometer.

Solid Phase Peptide Synthesis: All peptide analogs were synthesized using standard Fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS) procedures on preloaded Fmoc-L-Lys-4-benzyloxybenzyl alcohol (Wang) resin (0.343 mmol/g) either manually or using a Liberty 1 automated peptide synthesizer (CEM Corporation). For manual synthesis of peptides, the resin (0.100 g) was first swelled in 2 mL dichloromethane (DCM) for 20 min at room temperature, and then drained. The resin was then washed twice with 2 mL dimethylformamide (DMF) for 2 min with shaking at 200 rpm. Fmoc removal was accomplished with treatment of the resin by 2 mL of 20% piperidine in DMF and shaking at 200 rpm for 7.5 min. This process was repeated once more, for a total of 15 min deprotection time. The resin was then washed twice with 2 mL DMF for 2 min with shaking at 200 rpm. Coupling solutions were prepared using 2 equiv. of the desired amino acid, 2 equiv. of *N,N,N′,N′*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), and 2 equiv. of *N,N*-Diisopropylethylamine (DIPEA) in 2.5 mL DMF. The coupling solution was added to the resin, and shaken for approximately 1 h at 200 rpm. Following completion of the coupling reaction, the resin was washed twice with DMF for 2 min at 200 rpm. For automated synthesis of peptides, the resin was swelled and washed using the same protocol as manual synthesis. Deprotection of amino acids was performed with 20% piperidine in DMF for 15 sec at 75 °C and 160 watts, followed by 75 sec at 90 °C and 30 watts. Coupling was performed using 5 equiv. of the Fmoc-protected amino acid, 5 equiv. HBTU, and 10 equiv. DIPEA for 5 min at 75 °C and 20 watts. Arginine residues present within the sequence were double coupled.

Final Cleavage and Precipitation: Following deprotection of the final residue, the resin was washed three times with 2 mL DCM with manual shaking for 1 min. The resin was dried with ether for 5 min, flushed, and allowed to sit for 10 min to air dry. A 5 mL solution of 2.5% water, and 2.5% triisopropylsilane (TIPS) in TFA was prepared in a 15 mL polypropylene centrifuge tube. Dry resin was transferred from the reaction vessel to the 15 mL tube containing the cleavage cocktail, and the tube was shaken for 3 h at 200 rpm. Following completion of the cleavage reaction, the resin was filtered through a cotton plug in a polypropylene syringe and washed with a small amount of TFA. The filtrate was collected in a 50 mL polypropylene centrifuge tube, and cold 50:50 diethyl ether:hexane, up to 50 mL total volume, was added. The solution was stores at -20 °C for 20 min to facilitate peptide precipitation, followed by centrifugation at 4,500 rpm for 5 min to pellet precipitated peptides. Following centrifugation, the 50:50 ether:hexane solution was removed, and the solid peptide product was re-dissolved in 1:1 ACN:water, frozen in a dry ice-acetone bath, and then lyophilized for a minimum of 24 h.

Peptide Purification by HPLC: Crude peptides were purified using RP-HPLC. The crude peptide was dissolved in 5 mL of 5-50% ACN in water (depending on peptide solubility) and purified in 4 mL portions on a Phenomenex Kinetex 5 μm C18 semi-preparative column (10 mm x 250 mm, 110 Å). Initially, crude peptides were purified with a linear gradient from 5% to 40% ACN in 46 min. After identifying the relative retention time of these peptides, a second preparatory run was performed using a 10% ACN gradient centered on the ACN concentration where peptide elution was observed, for example a 15% to 25% ACN gradient for a peptide that eluted at 20% ACN during the first run. These conditions were typically sufficient to purify the peptides to ≥ 90-95%. Fraction purity was determined through analysis on a Phenomenex Kinetex 5 μm analytic C18 column (4.6 mm x 250 mm, 110 Å). The gradient used for analytical analysis was from 5% to 95% ACN over 24 min. Following purification, peptides were frozen with a dry ice-acetone bath, and then lyophilized for a minimum of 24 h. Before the final masses and purified yield of peptides was determined, peptides were dissolved in 25% acetic acid in up to 1:1 ACN:water to remove any residual TFA. The solution was then frozen and lyophilized for at least 24 h before peptide DMSO stocks were made for bioassays.

Peptide Verification with Mass Spectrometry: Following purification of crude peptide, peaks were verified to contain the desired peptide mass by MALDI-TOF MS. Samples were prepared using 1 μL α-Cyano-4-hydroxycinammic acid in 1:1 water:ACN as a matrix and 1 μL of the desired peptide fraction. Final verification of the peptide mass was conducted by obtaining their exact masses with a high resolution ESI-TOF MS (**Tables S-2 – S-3**). The instrument was calibrated before each run and an internal reference mass standard was used.

Isolation of Crude Peptides from Bacterial Supernatants: Overnight *S. oligofermentans* culture (200 mL) was centrifuged at 4,500 rpm for 10 min. The supernatants were fractionated and filtered through a sterile 0.22-μm polyethersulfone (PES) filter into sterile 50 ml centrifuge tubes. Ammonium sulfate was added to the supernatants to afford a 40% (wt/vol) concentration and the solutions were mixed by inversion until the ammonium sulfate was completely dissolved. The solution was stored at 4 °C for 1 hour, followed by centrifugation at 4,500 rpm for 15 min. The supernatants were then carefully decanted from the centrifuged tubes and the remaining pellet was dissolved in 10 mL of ddH_2O:ACN (1:1), after which all of the fractions were combined into a single centrifuge tube and freeze-dried. The lyophilized material was then purified by RP-HPLC.

Following purification, the mass of the extracted peptide was verified using a high resolution ESI-TOF MS and through MS/MS analysis (See **Figure S-7**).

Development of *S. oligofermentans* reporter strain:

Bacterial gDNA extraction: A single colony of *S. oligofermentans* was grown at 37 °C with 5% CO_2 in 10 mL THY media (pH 7.3) for 18 hours. Following incubation, 1 mL of pure culture broth was added to a sterile 1.5 mL microcentrifuge tube. Cells were pelleted by centrifugation at 10,000 rpm for 1 min. The supernatant was then discarded, and 1 mL 1x phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH adjusted to 7.2 - 7.4) was added to the tube containing the bacterial cell pellet. The pellet was resuspended using 5 s pulses with a vortex mixer set at 4,000 rpm. The cells were pelleted again under the same centrifugation conditions as above. The supernatant was discarded, and the process was repeated twice more for a total of three times. Following the PBS washes, 0.5 mL of sterile distilled water was added to the bacterial pellet, and it was resuspended by pulse vortex mixing at 4,000 rpm. The cells were then pelleted under the same conditions. The supernatant was discarded, and 250 μL of sterile distilled water was added to the tube. The pellet was again resuspended by vortex mixing at 4,000 rpm, incubated in a standard heatblock set at 95 °C for 5 min, and immediately placed in a −80 °C freezer for 10 min. After 10 min, the tubes were left at room temperature to thaw completely and mixed with a vortex mixer set at 4,000 rpm using 5 s pulses, followed by centrifugation at 5,000 rpm for 60 s. The supernatant was then transferred to a new, sterile, 0.6 mL microcentrifuge tube and stored at −80 °C.

S. oligofermentans comX promoter amplification: The promoter region of the *S. oligofermentans comX* gene was amplified from extracted bacteria gDNA using the primer pair SOComXfwd-SOComXrev (**Table S-1**) on an Eppendorf Mastercycler Gradient 5331 PCR machine. *comX* PCR was performed using 25 μL Hot Start Taq Master Mix (VWR), 5 μL template DNA, 10 μL of 5 μM primer SOComXfwd, and 10 μL of 5 μM primer SOComXrev, for a total reaction volume of 50 μL. PCR amplification consisted of the following steps: 3 min initial denaturation at 94 °C to activate the polymerase, followed by 30 cycles of 30 sec denaturation at 94 °C, 45 sec of annealing at 55 °C, and 60 sec of extension at 72 °C, followed by a final extension at 72 °C for 5 min. Following amplification, PCR products were ran on a 1% agarose gel to verify the presence of the *comX* PCR amplicon containing ends homologous to the restriction sites for *Bam*HI and *Nhe*I (~900 bp). Once verified, PCR products were purified using a E.Z.N.A Cycle Pure Kit (Omega Bio-Tek).

Table S-1: Primers used for the design of the *S. oligofermentans* luciferase reporter

Primer	Sequence
SOComXfwd	aaagctagcCAGTTTCAGAGCGTGATCGT[a]
SOComXrev	aaaggatccTCCACTGCACTTTCTCATAG[b]

[a]BamHI restriction site underlined. [b]NheI restriction site underlined.

Restriction digestion: The plasmid pFW11-*luc* ($Spec^R$) was extracted and purified from *Escherichia coli* using a GenElute Plasmid Miniprep Kit (Sigma). Both pFW11-*luc* and the PCR amplified *comX* promoter amplicon were then restriction digested using 500 ng DNA, 5 μL CutSmart Buffer (NEB), 1 μL *Nhe*I (NEB), 1 μL *Bam*HI-HF (NEB) and water to bring the total reaction volume up to 50 μL. The restriction digestion was ran for 1 h at 37 °C, followed by heat

inactivation of the restriction enzymes at 65 °C for 20 min. Following restriction digestion, digested products were ran on a 1% agarose gel to verify the presence of bands with a different size than undigested plasmid or PCR amplified *comX* promoter, respectively. Following verification of the correct amplicon, digested *comX* promoter was purified using a E.Z.N.A Cycle Pure Kit (Omega Bio-Tek). Restriction digested pFW11-*luc* ($Spec^R$) was purified via the addition of 1/10 volumes 3M sodium acetate and 2.5 volumes EtOH to the reaction. These components were mixed to homogeneity, and then centrifuged at 4 °C at 12,000 rpm for 15 min. The supernatant was then decanted, and the pelleted digested plasmid was washed with 70% EtOH, followed by centrifugation for 5 min at 4 °C and 12,000 rpm. The supernatant was then decanted, and the pellet was allowed to completely air dry, followed by resuspension in 20 μL TE Buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA).

Ligation and cloning: Purified restriction digestion pFW11-*luc* ($Spec^R$) and PCR amplified *comX* promoter were ligated together at an insert to vector ratio of 3:1, with the addition of 2 μL T4 DNA ligase buffer (NEB), 1 μL T4 DNA ligase (NEB), and nuclease water to bring the total reaction volume to 20 μL. Ligation of the insert and vector was performed at 16 °C overnight. Following ligation, the ligated construct was transformed into competent *E. coli.* Competent *E. coli* were removed from a -80 °C freezer, thawed in hand, and immediately placed on ice for 10 min. Ligated construct to be transformed was added at a concentration of 10 ng per 50 μL of competent cells, with the total volume of added DNA not exceeding 5% that of the competent cells. Tubes containing the competent *E. coli* and construct DNA were gently inverted several times to mix contents to homogeneity, after which they were placed on ice for 30 min. The tubes were then placed in a 42 °C heat bath for 90 sec, followed by immediate transfer to an ice bath where they were cooled for 2 min. 400 μL of SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added to the tube, which was then transferred to a 37 °C shaking incubator set at 200 rpm for 45 min. Following incubation, the entire content of the tube was transferred to a LB agar plate containing 50 μg/mL spectinomycin, after which the liquid was evenly spread and allowed to soak into the plate surface. The plate was then incubated at 37 °C overnight, and was checked for the presence of positive transformants the following day.

Construct transformation: A single colony from plates containing successfully transformed competence *E. coli* was inoculated into 10 mL of LB media containing 50 μg/mL spectinomycin, which was then grown overnight at 37 °C with shaking at 200 rpm. Purified plasmid construct was extracted from these *E. coli* overnights using a GenElute Plasmid Miniprep Kit (Sigma). To verify the presence of the correct vector and insert, a small amount of the extracted plasmid was subjected to restriction digestion by *Bam*HI and *Nhe*I using the above conditions. Successful constructs were identified as containing two bands corresponding to the approximate size of the vector and insert when run on a 1% agarose gel. Following construct verification, a single colony of *S. oligofermentans* was inoculated into 10 mL THY media and grown for 18 h at 37 °C with 5% CO_2. The overnight culture was then diluted 1:25 in fresh THY media, followed by 2 h growth at 37 °C with 5% CO_2. Following incubation, exogenous synthetic CSP (10,000 nM) and plasmid construct (400 ng) were added to the diluted culture, and the bacteria were subjected to another 2 h growth at 37 °C with 5% CO_2. A 200 μL portion of the incubated bacteria was then plated and evenly spread across the surface of a THY plate containing 400 μg/mL spectinomycin. The liquid was allowed to absorb into the surface of the plate at RT, and then plated bacteria were grown at 37 °C with 5% CO_2 until bacterial colonies could be visualized (72 hrs). A single colony was then picked and grown for 18 hrs in 10 mL THY media containing 100 μg/mL spectinomycin at 37 °C with

5% CO_2. Following overnight growth, the *S. oligofermentans* culture was diluted 1:10 in fresh THY media, and the presence of the reporter plasmid was validated by both sequencing of the *comX* promoter region and the observation of luminescence following treatment with exogenous CSP (10,000 nM) and D-luciferin.

Biofilm Formation Assay: A single colony of *S. oligofermentans* was grown for 18 hours in 10 mL THY media (pH 7.3) at 37 °C with 5% CO_2. Following incubation, *S. oligofermentans* was diluted 1:100 in fresh THY media containing 1% D-glucose, and 198 µL was added in triplicate to a 96-well microtiter plate. Each well contained either 2 µL of the desired concentration of peptide in DMSO, or just DMSO as a negative control. A set of wells containing only THY media containing 1% D-glucose and DMSO (no bacteria) was included for background subtraction. The plate was then statically incubated at 37 °C with 5% CO_2 for 24 hours, and following incubation the absorbance at 600 nm (A600) was recorded. The contents of all wells were carefully decanted by shaking the plate gently over a glass basin. Experimental wells were then gently washed three times with 250 µL 1x PBS. To heat fix bacterial biofilms to the bottom of the well, the 96 well microtiter plate was incubated at 55 °C for 2 h. Following heat fixing, 200 µL of a 0.1% crystal violet solution was added to each well, and the solution was allowed to rest at room temperature for 5 min. The wells were then carefully decanted and washed twice with 200 µL water, and a total of 200 µL of a 30% (v/v) acetic acid in water was added to the wells. The plate was shaken for 15 min at 37 °C, and experimental wells were then diluted 1:5 in water. The absorbance at 595 nm (A595) was then measured for each well. Each A595 value was divided by its corresponding A600 values. Experiments were performed in triplicate on three separate days. Data is presented as the percent biofilm formation relative to wild type untreated with exogenous CSP. Results are expressed as the mean +/- the standard deviation of three independent experiments.

Hydrogen Peroxide Production Assay: A single colony of *S. oligofermentans* was grown overnight at 37 °C with 5% CO_2. The overnight culture was then diluted 1:100 in THY media containing 10 mM L-lactic acid, and 198 µL was added in triplicate to the wells of a 96-well microtiter plate. Each well contained 2 µL of the desired concentration of peptide in DMSO or just DMSO as a negative control. A set of three wells containing only the THY media with 10 mM L-lactic acid and DMSO (no bacteria) was included as an additional control. Plates were statically incubated for 24 hours at 37°C under 5% CO_2, after which the absorbance at 600 nm (A600) was recorded. To isolate the bacterial supernatant, plates were centrifuged at 4,000 rpm for 5 min. Following centrifugation, 104 µL of the culture supernatant was added to wells on a fresh plate with 96 µL of a solution containing 2.5 mM 4-aminoantipyrine (4-amino-2, 3-dimethyl-1-phenyl-3-pyrazolin-5-one) and 0.17 M phenol, giving a total volume of 200 µL in each well. The reaction was allowed to proceed for 4 min at room temperature, after which horseradish peroxidase in 0.2 M potassium phosphate buffer (pH 7.2) was added at a concentration of 50 mU/ml. Following a second 4 min incubation, the absorbance at 510 nm (A510) was measured. A standard curve was generated using the same protocol with known concentrations of H_2O_2 in THY media (**Figure S-1**). Experimental A510 values were fitted to the slope of the standard curve, and H_2O_2 concentrations in each sample were determined. Experiments were performed in triplicate on three separate days. Data is presented as the percent H_2O_2 production relative to wild type untreated with exogenous CSP. Results are expressed as the mean +/- the standard deviation of three independent experiments.

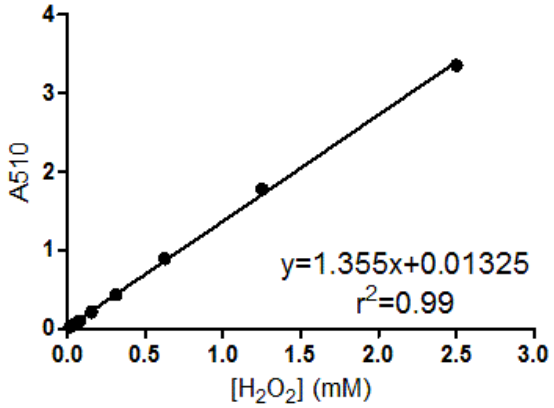


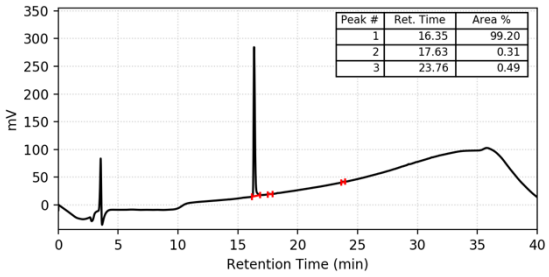
Figure S-1. Calibration curve correlating serial dilutions of H_2O_2 to A510 values. Experiment was performed in triplicate.

Hemolysis Assay: A single colony of *S. oligofermentans* was grown for 18 h at 37 °C with 5% CO_2 in 10 mL THY media (pH 7.3). Following incubation cultures were diluted 1:25 in fresh THY media. Samples were then prepared in clear bottom 96-well microtiter plates. Experimental samples were prepared by adding 198 μL of the 1:25 dilution and 2 μL of a 1 mM CSP stock, or 198 μL of fresh THY media and 2 μL of a 1 mM CSP stock. A positive control was prepared by adding 2 μL of a 1% Triton X solution in 198 μL THY media, and negative controls were prepared by adding 2 μL DMSO in either 198 μL 1:25 dilution or 198 μL THY. The 96-well microtiter plate was the incubated for 5 h at 37 °C with 5% CO_2, after which the A600 was recorded and hemolysis was assessed. A 1 mL portion of defibrinated rabbit red blood cells (Thermofisher) was aliquoted into a sterile 1.5 mL microcentrifuge tube, and centrifuged at 2,000 rpm for 2 min. Following centrifugation, the top layer was aliquoted off, and red blood cells were gently washed with 1 mL PBS. This process was repeated for a total of three times, until following centrifugation the top layer was mostly clear. Washed red blood cells were resuspended in 1 mL PBS, and a 15 μL aliquot was added to each well of the 96-well microtiter plate. The plate was then incubated for 30 min at 37 °C. Following incubation, the plate was centrifuged for 4 min at 4 °C at 1,600 rpm, and a 150 μL portion of the resulting supernatant was carefully removed and placed in a fresh 96-well microtiter plate. Experimental wells were then diluted 1:5 in water to prevent saturation of the detector by the positive control. The A420 for each experimental well was then recorded. Experiments were performed in triplicate on three separate days. Data is presented as the percent hemolysis relative to the .01% Triton X positive control. Results are expressed as the mean +/- the standard deviation of three independent experiments.

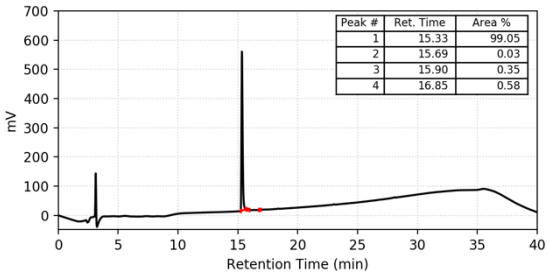
HPLC Traces for Peptide Analogs

HPLC Traces for *S. oligofermentans* CSP Alanine Screen Analogs

S. oligofermentans CSP



S. oligofermentans CSP D1A



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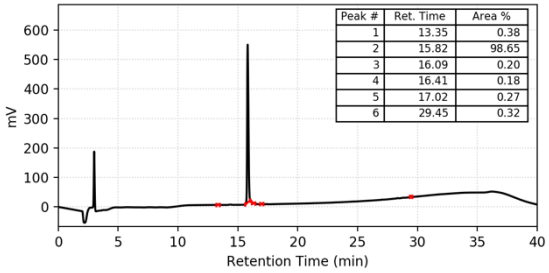
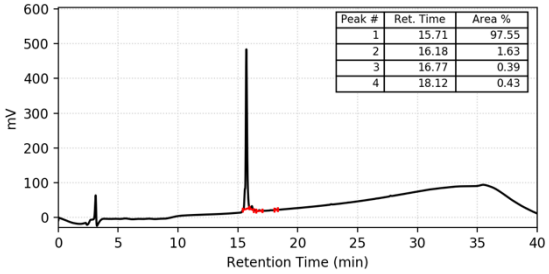


S. oligofermentans C SP R3A



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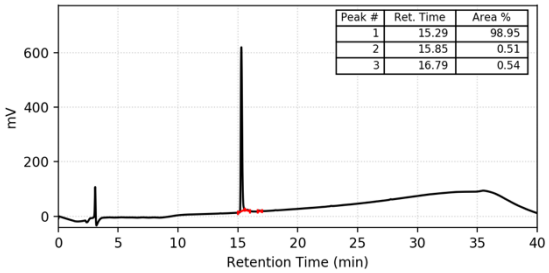

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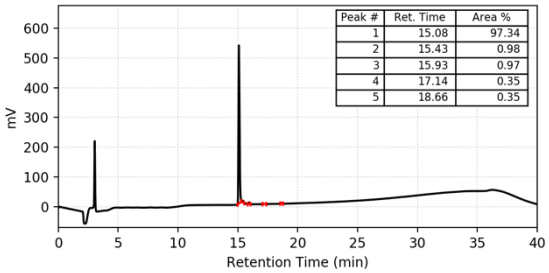

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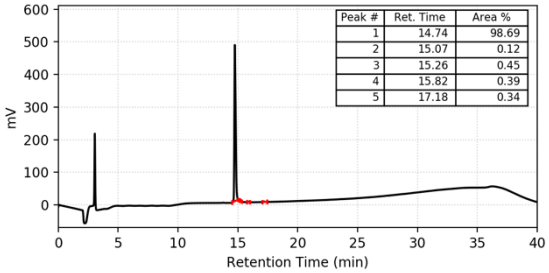

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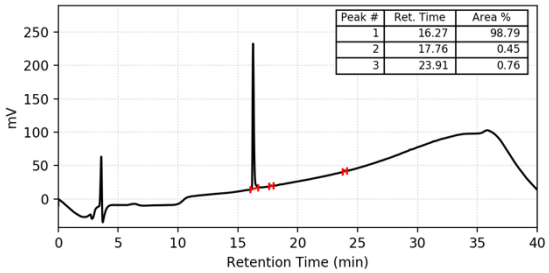

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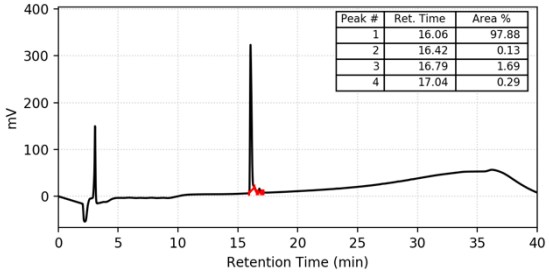
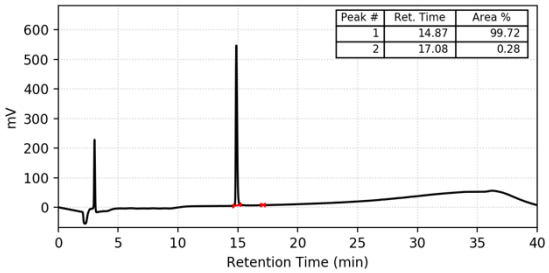
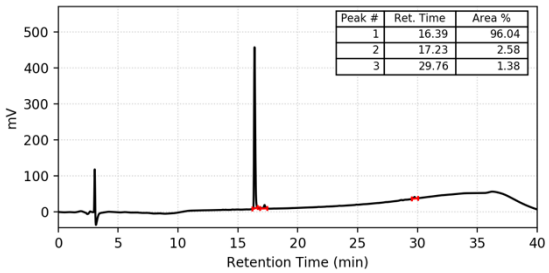


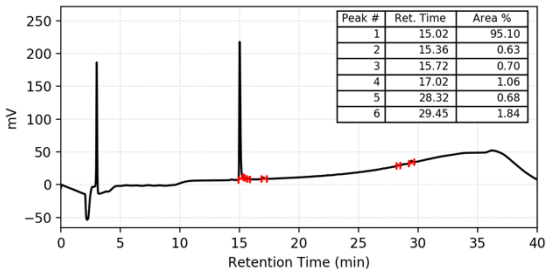
S. oligofermentans CSP I9A



S. oligofermentans CSP K10A



S. oligofermentans CSP F11A



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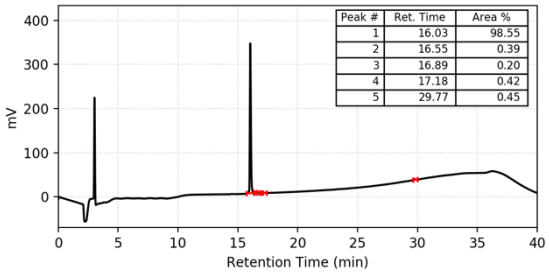

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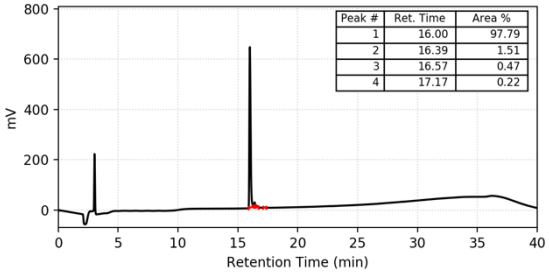
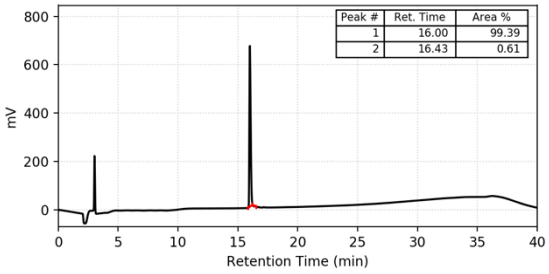
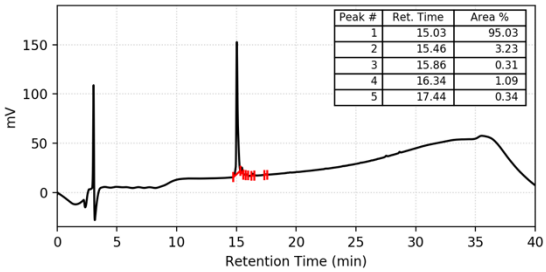


S. oligofermentans CSP K14A



HPLC Traces for *S. oligofermentans* CSP Truncation & Double Mutation Analogs

S. oligofermentans CSP-des-D1



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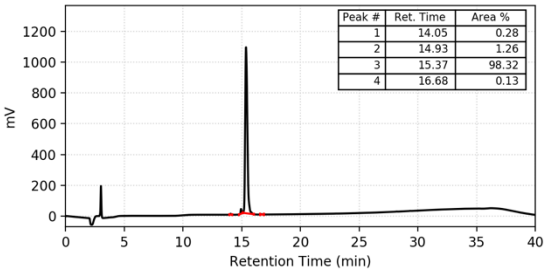
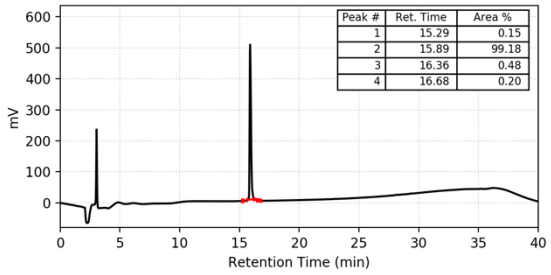
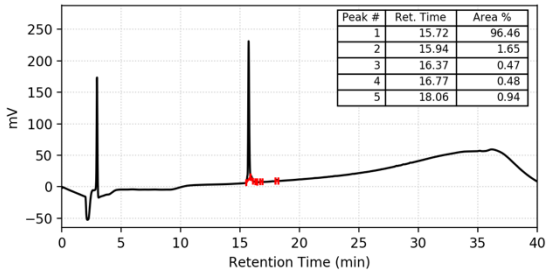


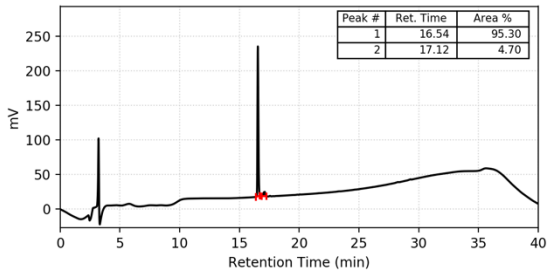
S. oligofermentans CSP-des-D1S2R3



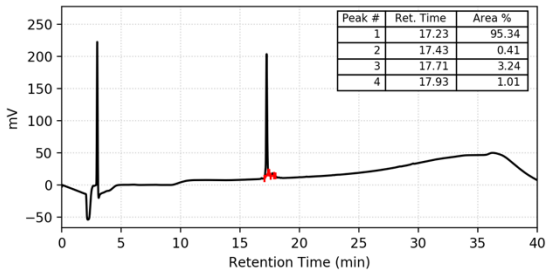
S. oligofermentans CSP-des-K14



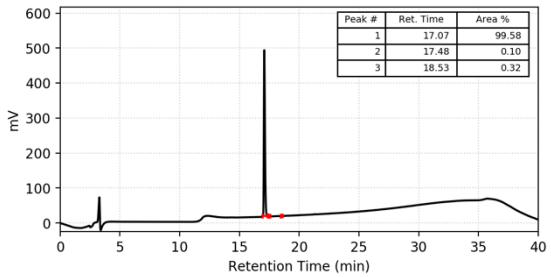
S. oligofermentans CSP-des-K13K14



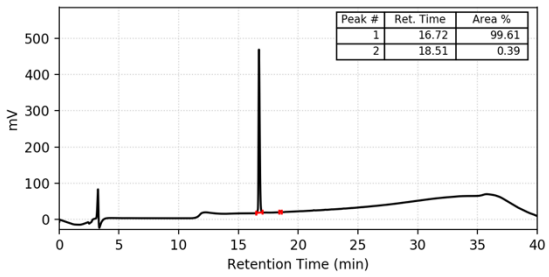
S. oligofermentans CSP-des-K12K13K14



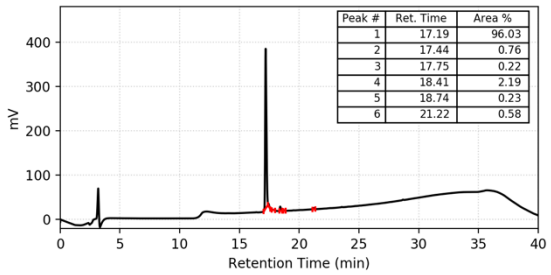
S. oligofermentans CSP D1AS2A



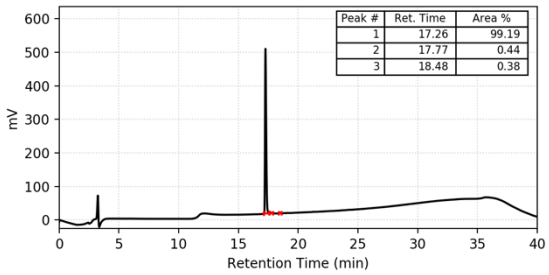
S. oligofermentans CSP D1AL7A



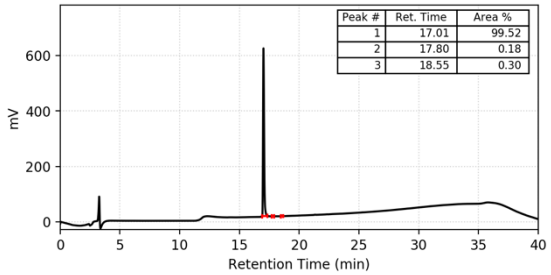
S. oligofermentans CSP D1AK13A



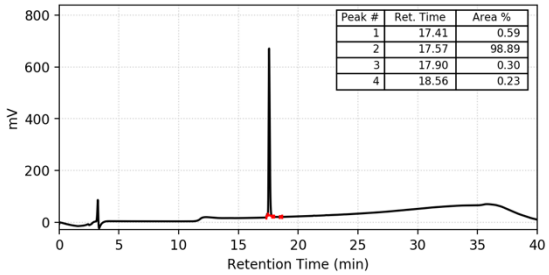
S. oligofermentans CSP D1AK14A



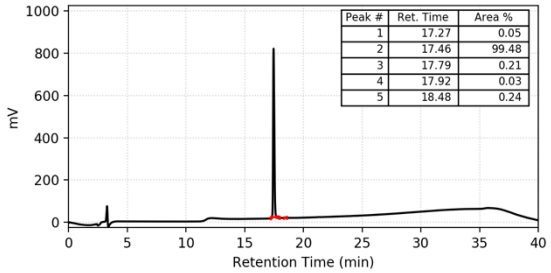
S. oligofermentans CSP S2AL7A



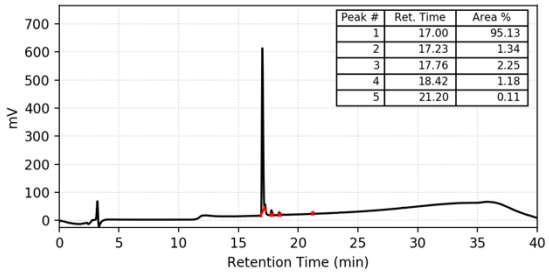
S. oligofermentans CSP S2AK13A



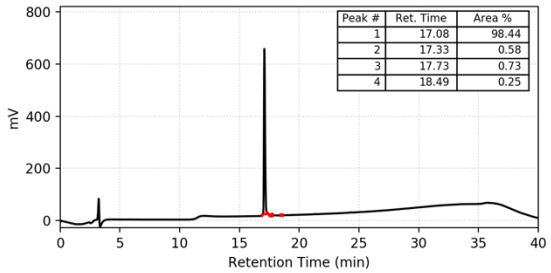
S. oligofermentans CSP S2AK14A



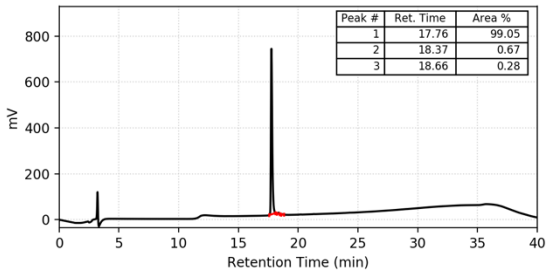
S. oligofermentans CSP L7AK13A



S. oligofermentans CSP L7AK14A



S. oligofermentans CSP K13AK14A



HRMS and HPLC Data for *S. oligofermentans* CSP Analogs

Table S-2. HRMS and HPLC Data for all *S. oligofermentans* CSP Alanine Screen Analogs

Peptide #	Peptide Name	Calc. EM Mass	Obs. EM Mass	Purity (%)
1	*S. oligofermentans* Native CSP	883.0459*	883.0460*	≥99%
2	*S. oligofermentans* CSP D1A	861.0511*	861.0511*	≥99%
3	*S. oligofermentans* CSP S2A	875.0485*	875.0461*	≥98%
4	*S. oligofermentans* CSP R3A	840.5139*	840.5121*	≥97%
5	*S. oligofermentans* CSP N4A	861.5431*	861.5420*	≥98%
6	*S. oligofermentans* CSP I5A	862.0225*	862.0264*	≥97%
7	*S. oligofermentans* CSP F6A	845.0303*	845.0307*	≥98%
8	*S. oligofermentans* CSP L7A	575.0174¤	575.0153¤	≥98%
9	*S. oligofermentans* CSP K8A	1708.0268†	1708.0315†	≥97%
10	*S. oligofermentans* CSP I9A	862.0225*	862.0258*	≥99%
11	*S. oligofermentans* CSP K10A	854.5171*	854.5139*	≥96%
12	*S. oligofermentans* CSP F11A	854.0303*	854.0331*	≥95%
13	*S. oligofermentans* CSP K12A	854.5171*	854.5142*	≥98%
14	*S. oligofermentans* CSP K13A	854.5171*	854.5198*	≥97%
15	*S. oligofermentans* CSP K14A	854.5171*	854.5202*	≥99%

EM = Exact Mass. See above for methods, †MH_1^{+}, *MH_2^{2+}, ¤MH_3^{3+}

Table S-3. HRMS and HPLC Data for all *S. oligofermentans* CSP Truncation & Double Mutation Analogs

Peptide #	Peptide Name	Calc. EM Mass	Obs. EM Mass	Purity (%)
16	*S. oligofermentans* CSP-des-D1	550.6908¤	550.6887¤	≥95%
17	*S. oligofermentans* CSP-des-D1S2	521.6801¤	521.6789¤	≥98%
18	*S. oligofermentans* CSP-des-D1S2R3	469.6464¤	469.6444¤	≥99%
19	*S. oligofermentans* CSP-des-K14	546.3348¤	546.3346¤	≥96%
20	*S. oligofermentans* CSP-des-K13K14	503.6364¤	503.6347¤	≥95%
21	*S. oligofermentans* CSP-des-K12K13K14	690.9035*	690.9002*	≥95%
22	*S. oligofermentans* CSP D1AS2A	853.0536*	853.0564*	≥99%
23	*S. oligofermentans* CSP D1AL7A	840.0276*	840.0274*	≥99%
24	*S. oligofermentans* CSP D1AK13A	832.5221*	832.5225*	≥96%
25	*S. oligofermentans* CSP D1AK14A	832.5221*	832.5227*	≥99%
26	*S. oligofermentans* CSP S2AL7A	1707.0428†	1707.0454†	≥99%
27	*S. oligofermentans* CSP S2AK13A	846.5196*	846.5177*	≥98%
28	*S. oligofermentans* CSP S2AK14A	846.5196*	846.5190*	≥99%
29	*S. oligofermentans* CSP L7AK13A	833.4936*	833.4942*	≥95%
30	*S. oligofermentans* CSP L7AK14A	833.4936*	833.4971*	≥98%
31	*S. oligofermentans* CSP K13AK14A	825.9881*	825.9910*	≥99%

EM = Exact Mass. See above for methods, †MH_1^{+}, *MH_2^{2+}, ¤MH_3^{3+}

Luciferase Reporter Gene Assay Data

Agonism assays were performed at a CSP concentration of 10,000 nM. The native *S. oligofermentans* CSP was used as a positive control while DMSO was used as the negative control. Percent *comX* activation was measured by normalizing the blank and OD_{600} corrected luminescence obtained for each peptide to that of the native *S. oligofermentans* CSP. All peptides were screened in triplicate in three separate trials. Error bars indicate the standard deviation from the mean of the nine separate values.

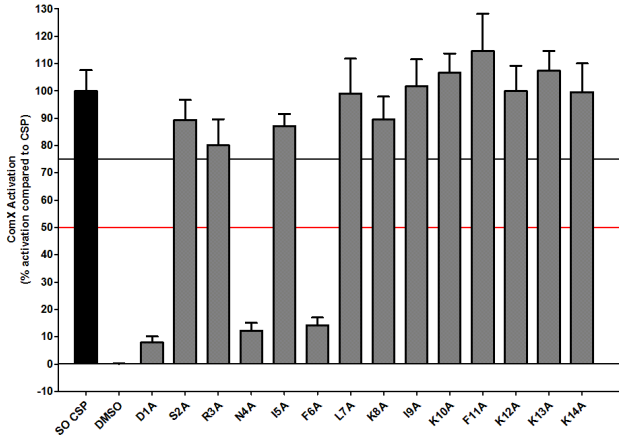


Figure S-2. Primary agonism screening assay data for the *S. oligofermentans* CSP alanine screening library. Peptides that exhibited over 75% activation were further evaluated to determine their EC_{50} values, while peptides that exhibited less than 50% activation were further evaluated for their potential as competitive inhibitors.

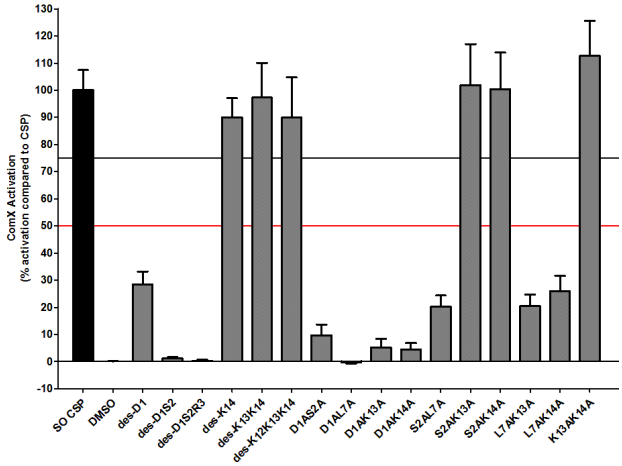


Figure S-3. Primary agonism screening assay data for the *S. oligofermentans* CSP truncation and double mutation screening libraries. Peptides that exhibited over 75% activation were further evaluated to determine their EC_{50} values, while peptides that exhibited less than 50% activation were further evaluated for their potential as competitive inhibitors.

Antagonism assays were performed at a CSP concentration of 10,000 nM against a 300 nM concentration of the native *S. oligofermentans* CSP. The native *S. oligofermentans* CSP (300 nM) was used as a positive control, while DMSO was used as a negative control. Percent *comX* activation was measured by normalizing the blank and OD_{600} corrected luminescence obtained for each peptide to that of the native *S. oligofermentans* CSP. All peptides were screened in triplicate in three separate trials. Error bars indicate the standard deviation from the mean of the nine separate values.

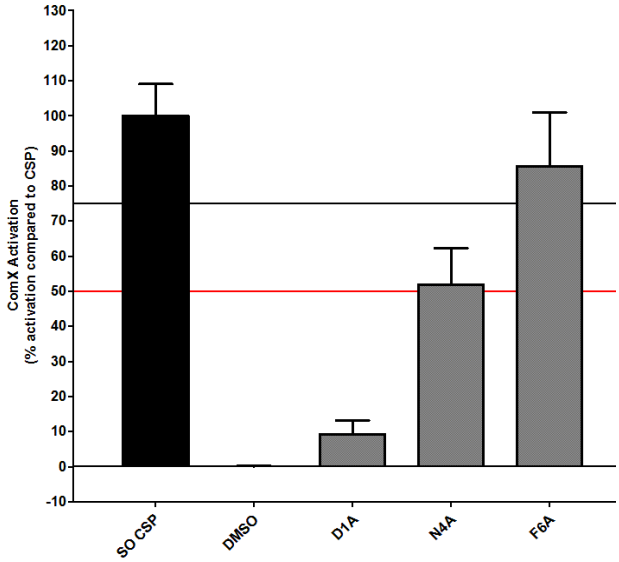


Figure S-4. Primary antagonism screening assay data for the *S. oligofermentans* CSP alanine screening library. Peptides that exhibited less than 50% activation were further evaluated to determine their IC_{50} values.

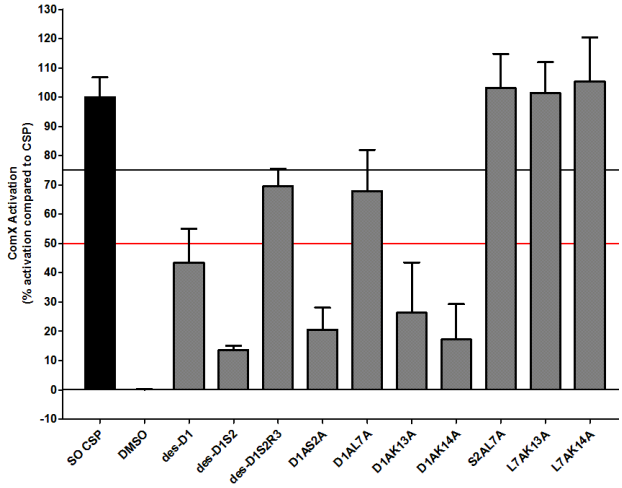
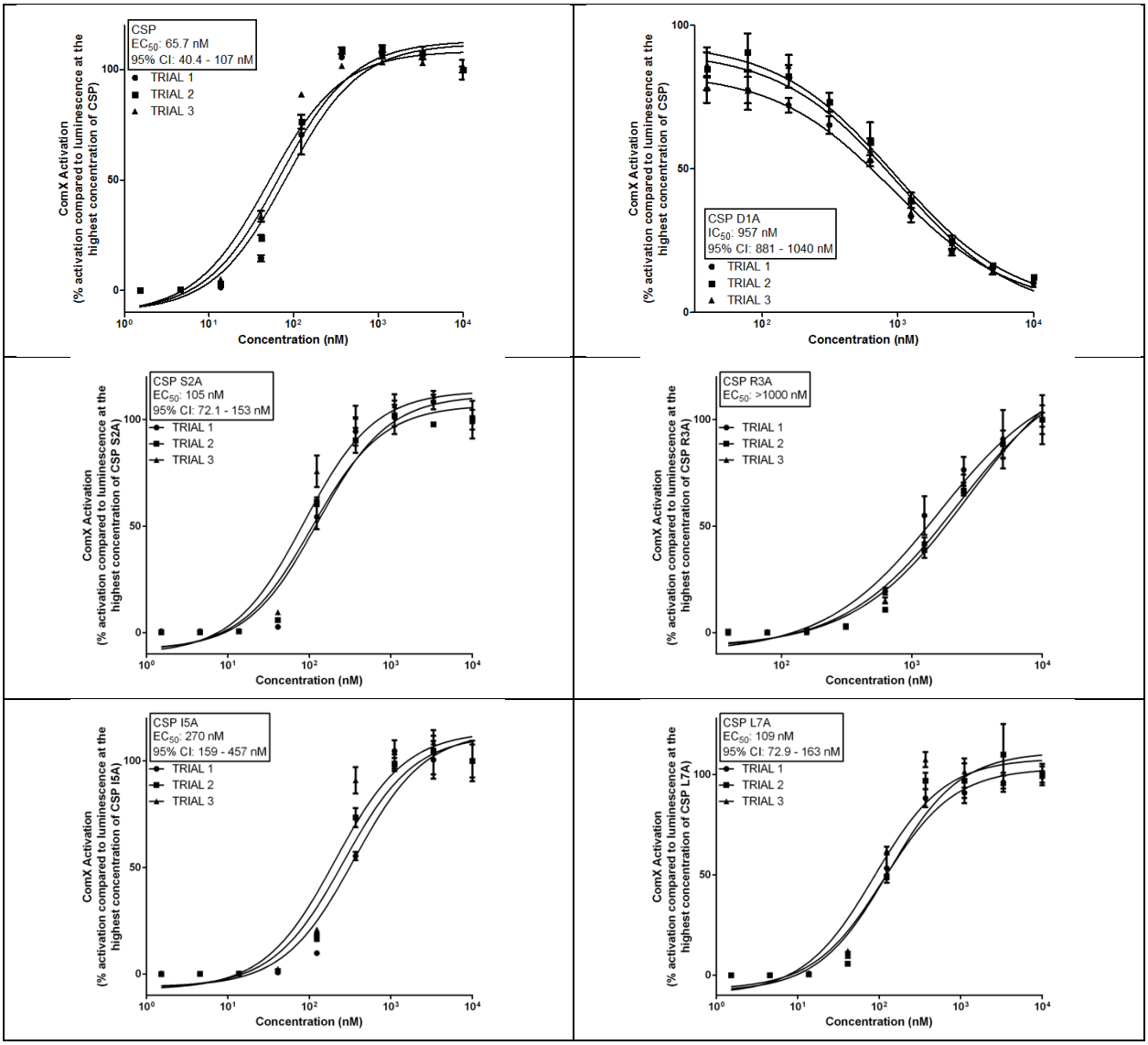


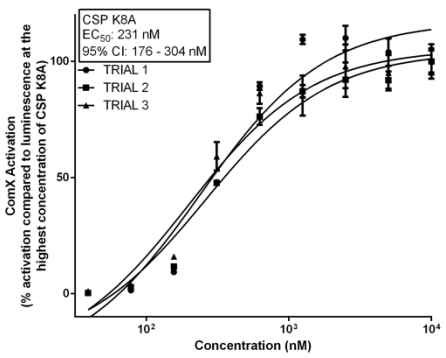
Figure S-5. Primary antagonism screening assay data for the *S. oligofermentans* CSP truncation and double mutation screening libraries. Peptides that exhibited less than 50% activation were further evaluated to determine their IC_{50} values.

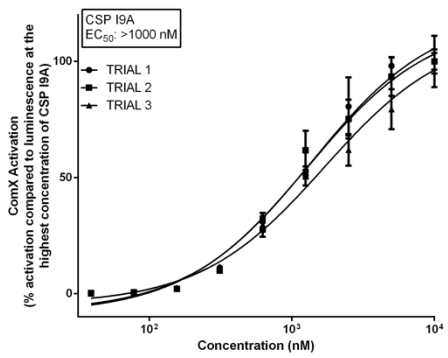
CSP Analog Agonism and Antagonism Dose Response Curves

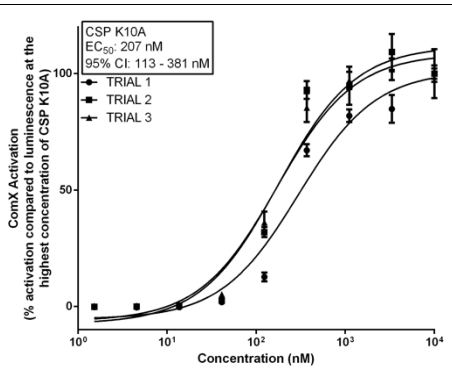
CSP analogs were screened over varying concentrations using the *S. oligofermentans* luminescence reporter strain. Each dose response curve was assessed in triplicate in three separate trials (i.e., trial 1-3 shown for each peptide below). Error bars indicate the standard deviation from the mean of the nine separate values. In each plot, the peptide and its EC_{50} or IC_{50} value and 95% confidence interval values (95% CI) are indicated in the top left.

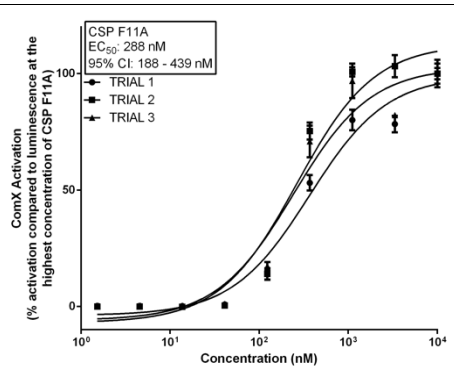
S. oligofermentans CSP Alanine Screen Analogs Dose Response Curves

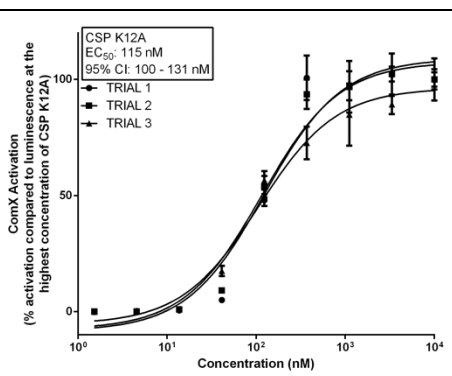


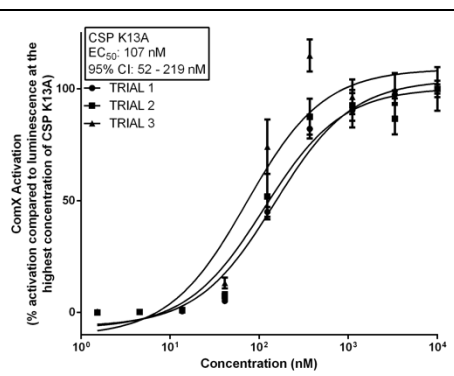

CSP K8A
EC50: 231 nM
95% CI: 176 - 304 nM
TRIAL 1
TRIAL 2
TRIAL 3
ComX Activation
(% activation compared to luminescence at the highest concentration of CSP K8A)
Concentration (nM)

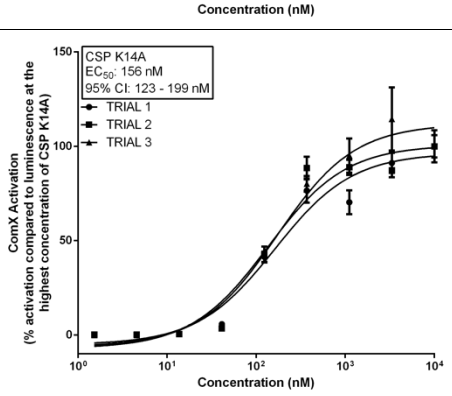

CSP I9A
EC50: >1000 nM
TRIAL 1
TRIAL 2
TRIAL 3
ComX Activation
(% activation compared to luminescence at the highest concentration of CSP I9A)
Concentration (nM)


CSP K10A
EC50: 207 nM
95% CI: 113 - 381 nM
TRIAL 1
TRIAL 2
TRIAL 3
ComX Activation
(% activation compared to luminescence at the highest concentration of CSP K10A)
Concentration (nM)

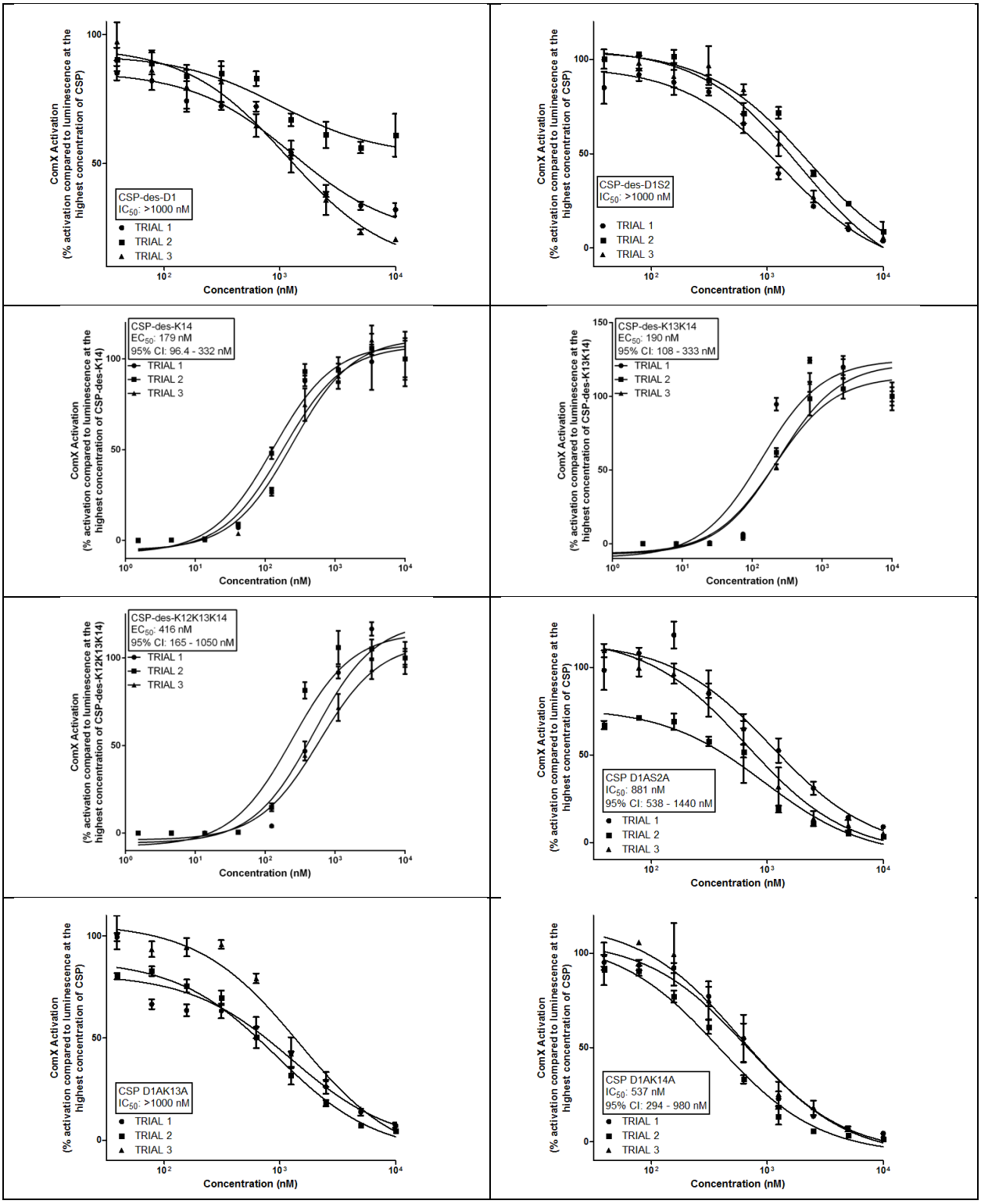

CSP F11A
EC50: 288 nM
95% CI: 188 - 439 nM
TRIAL 1
TRIAL 2
TRIAL 3
ComX Activation
(% activation compared to luminescence at the highest concentration of CSP F11A)
Concentration (nM)

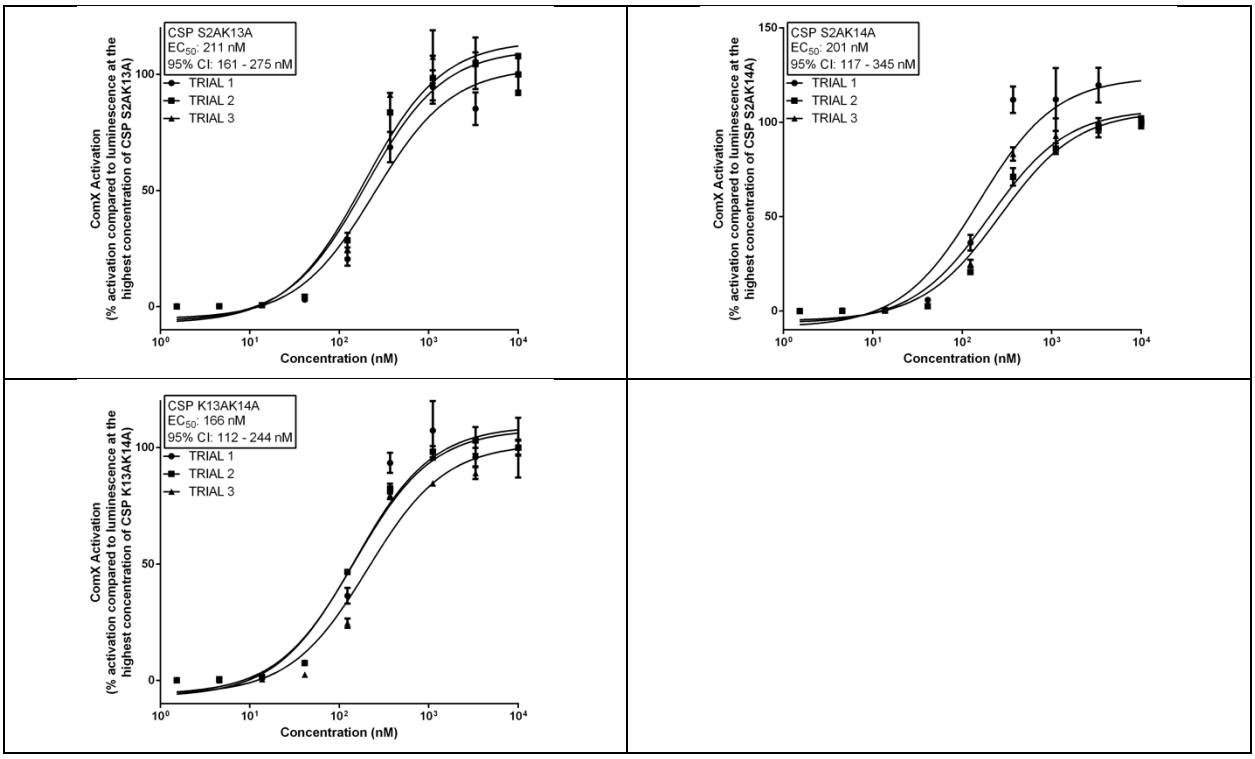

CSP K12A
EC50: 115 nM
95% CI: 100 - 131 nM
TRIAL 1
TRIAL 2
TRIAL 3
ComX Activation
(% activation compared to luminescence at the highest concentration of CSP K12A)
Concentration (nM)


CSP K13A
EC50: 107 nM
95% CI: 52 - 219 nM
TRIAL 1
TRIAL 2
TRIAL 3
ComX Activation
(% activation compared to luminescence at the highest concentration of CSP K13A)
Concentration (nM)


CSP K14A
EC50: 156 nM
95% CI: 123 - 199 nM
TRIAL 1
TRIAL 2
TRIAL 3
ComX Activation
(% activation compared to luminescence at the highest concentration of CSP K14A)
Concentration (nM)

S. oligofermentans CSP Truncation & Double Mutation Analogs Dose Response Curves





CD Spectra of Alanine Screen Peptides in PBS Buffer

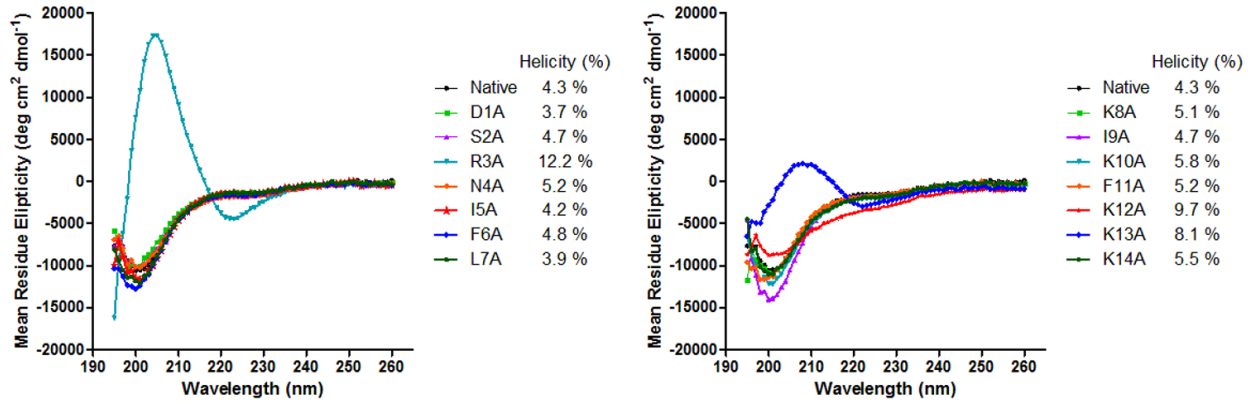
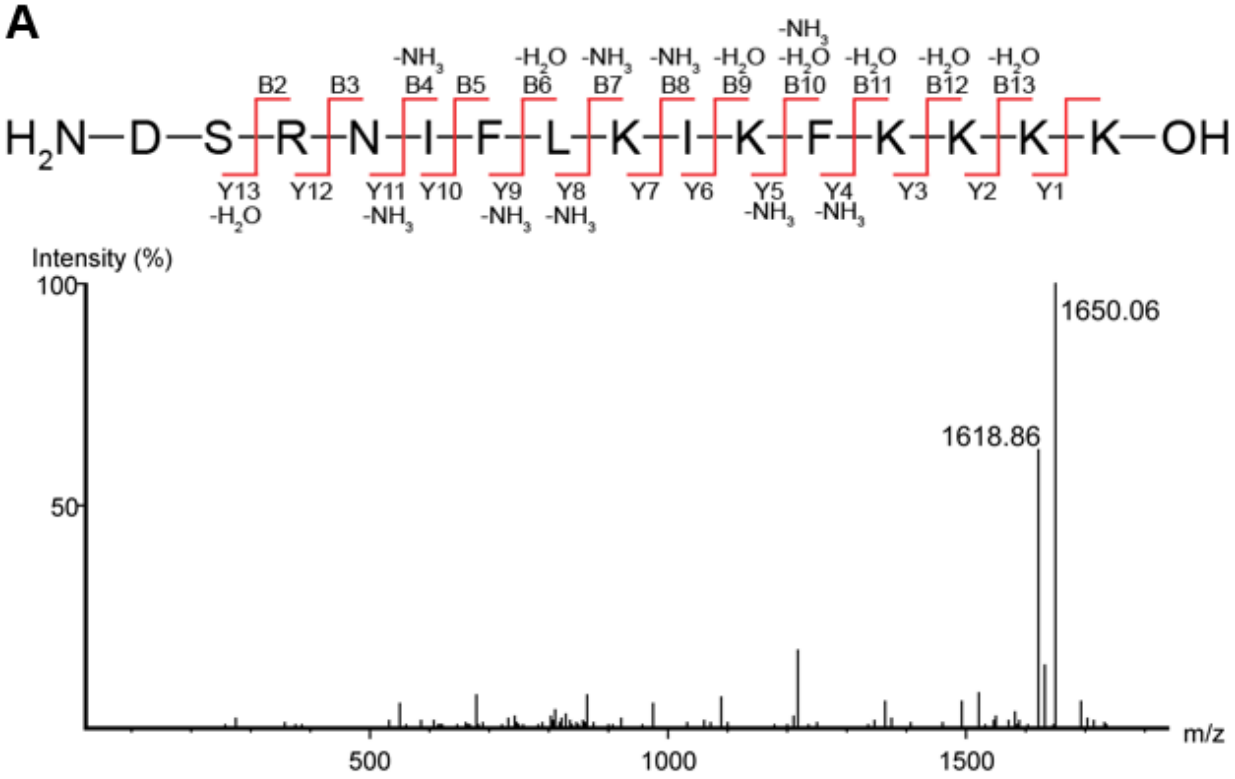


Figure S-6. CD spectra of *S. oligofermentans* alanine screen analogs in PBS buffer. Most of the alanine screen analogs adopt a random coil conformation, with the exception of R3A and K13A, which are beta sheets.

A



B

B	B Ions	B-H2O	B-NH3	AA	Y-NH3	Y-H20	Y Ions	Y
1	--	--	--	D	--	--	--	14
2	203.066	185.056	--	S	1633.03	1632.05	1650.0577	13
3	359.167	341.157	342.1408	R	1546	--	1563.0257	12
4	473.21	455.2	456.1837	N	1389.9	--	1406.9246	11
5	586.294	568.284	569.2678	I	1275.86	--	1292.8817	10
6	733.363	715.352	716.3362	F	1162.77	--	1179.7976	9
7	846.447	828.436	829.4203	L	1015.7	--	1032.7292	8
8	974.542	956.531	957.5152	K	902.619	--	919.6451	7
9	1087.63	1069.62	1070.5993	I	774.524	--	791.5502	6
10	1215.72	1197.71	1198.6943	K	661.44	--	678.4661	5
11	1362.79	1344.78	1345.7627	F	533.345	--	550.3711	4
12	1490.88	1472.87	1473.8576	K	386.276	--	403.3027	3
13	1618.98	1600.97	1601.9526	K	258.181	--	275.2078	2
14	--	--	--	K	--	--	147.1128	1

Figure S-7. MS/MS analysis of the extracted *S. oligofermentans* CSP. (A) *S. oligofermentans* CSP sequence showing the various MS/MS fragments detected in the raw spectrum. (B) Complete table of expected masses for the MS/MS analysis of the predicted *S. oligofermentans* CSP with highlighted masses correspond to ion peaks detected.