Enhancement of amino acid production and secretion by *Lactococcus lactis* using a droplet-based biosensing and selection system

Hernandez-Valdes *et al*.

Supplementary Material

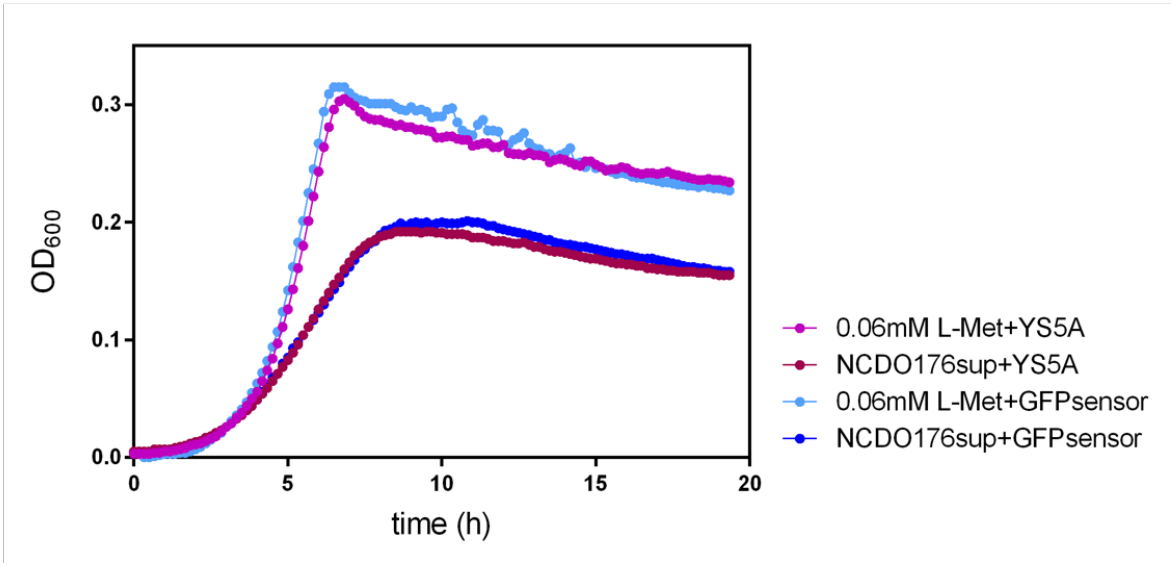

Fig. S1. The GFPsensor and YS5A strains show similar growth to assess amino acid secretion. The oligopeptide transport system in *L. lactis* consists of Opp, DtpT and Dpp. The strain GFPsensor is a deletion mutant of *opp* and *dtpT,* whereas YS5A is a deletion mutant of *opp, dtpT, dpp.* Previous studies in *L. lactis* MG1363 have reported that the *dppP* gene is not functional because of a nonsense mutation and a frameshift (Doeven et al., 2005). Therefore the *dpp* does not play a role in the peptide transport in the MG1363 strain. Both strains were tested for their capacity to grow in CDM-casein supplemented with a bacterial supernatant containing essential amino acids (NCDO176sup; dark blue and dark pink), and in a growth medium where the essential amino acid methionine was removed (CDM-met) and supplemented with methionine at a concentration of 0.06 mM (light blue, and light pink). Both strains show similar growth rate and identical cell density (OD_{600}) in each growth condition. Thus, the GFPsensor that lacks two peptide transport systems is suitable as sensor cell.

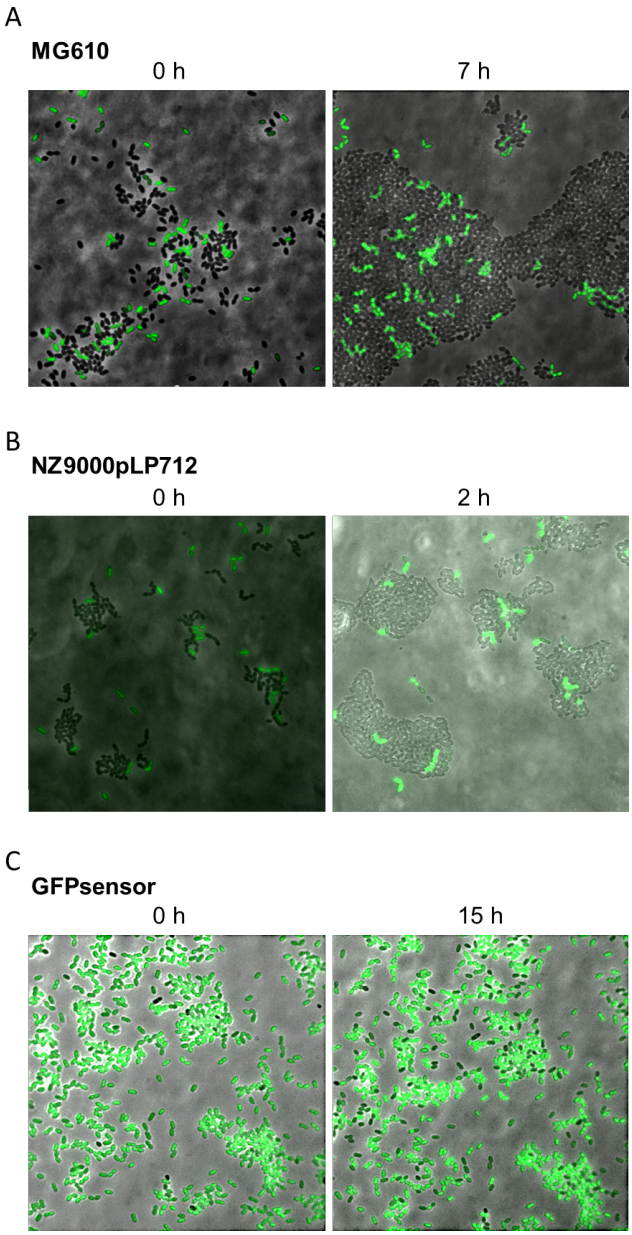

Fig. S2. The GFPsensor is unable to grow in co-cultivation with the PrtP+ strains MG610 and NZ900pLP712. Snapshots of time-lapse experiments of the GFPsensor on CDM-casein show no growth of this strain. (A) Co-cultivation of GFPsensor and MG610 in CDM-casein, the GFPsensor cells are unable to grow after incubation (right image). (B) Co-cultivation of NZ9000pLP712 and GFPsensor on CDM-casein shows casein precipitation (formation of opaque and white layer), but the GFPsensor cells are also unable to grow. (C) Control sample of non-growing GFPsensor cells. Overlays of fluorescence-channel and bright-field are shown.

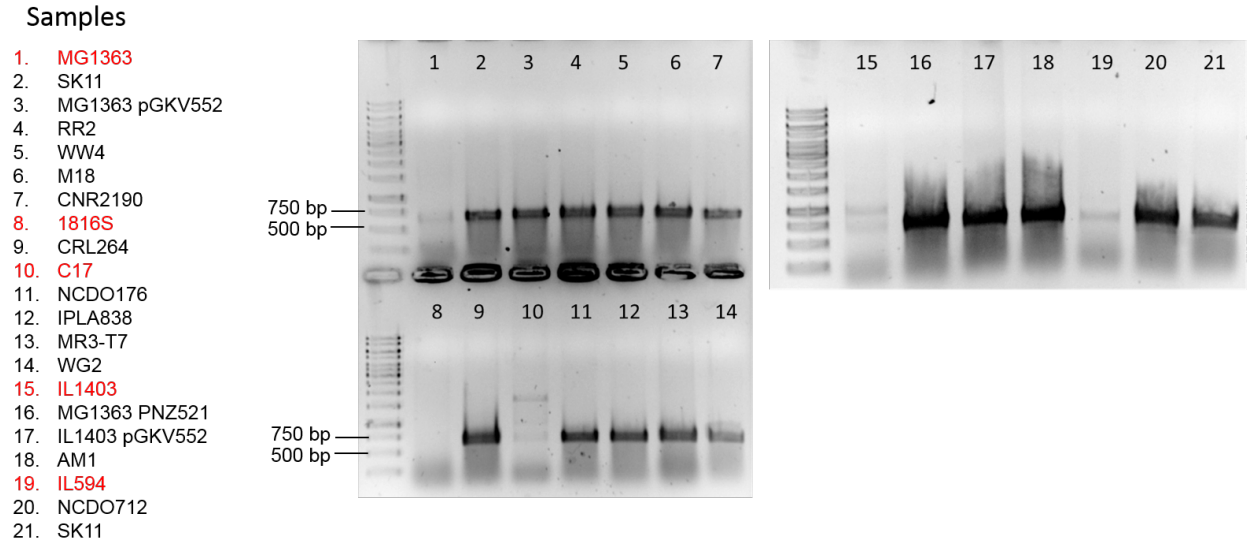

Fig. S3. Confirmation of the presence of *prtP* gene in *L. lactis* strains by PCR. The presence of the proteinase gene *prtP* was confirmed by PCR amplification, using the primers TestPrtP_Fw1 and TestPrtP_Rv1, which amplify a fragment of 604 bp. Samples indicated in red are PrtP negative (PrtP-), some of them such as the laboratory strains MG1363 and IL1403 were added as negative control.

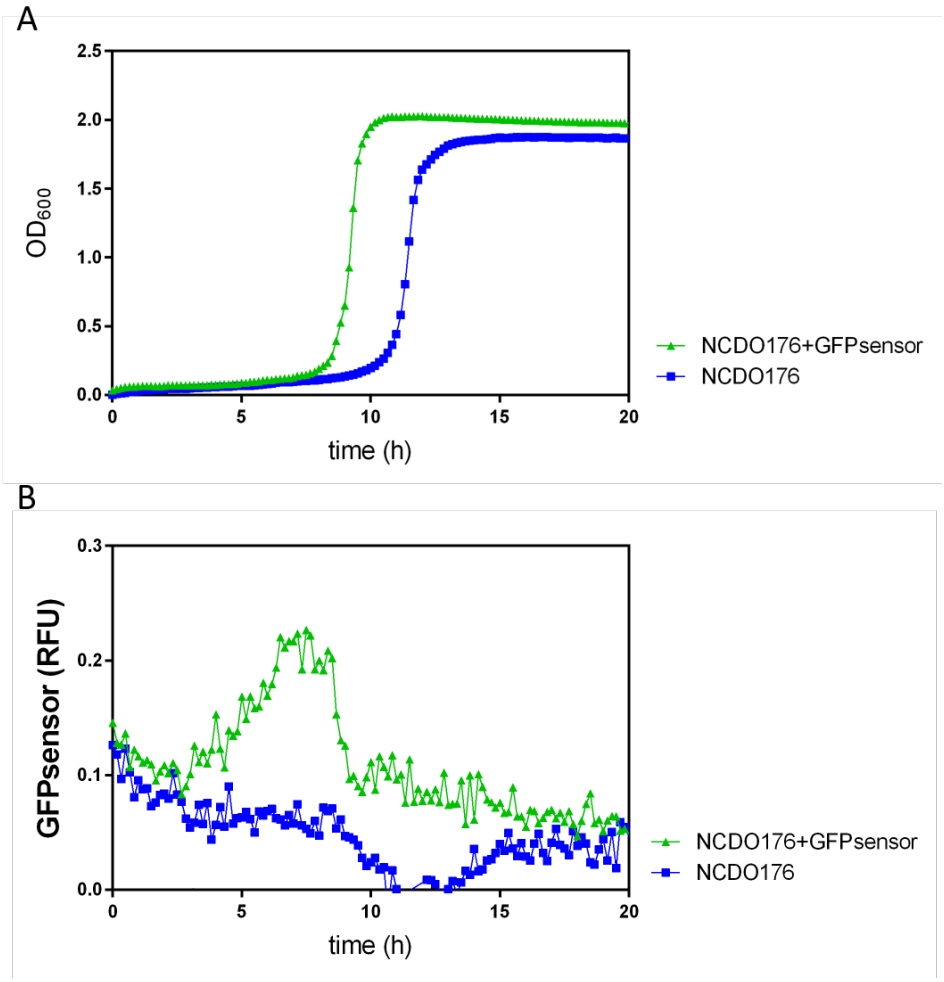

Fig. S4. Plate reader assay for GFPsensor signal measurements obtained by co-cultivation of producer strains with GFPsensor strain. (A) Growth curve of NCDO176 strain, as a single culture (blue line) and in co-cultivation with GFPsensor (green line), in a ratio 1:10 (producer:sensor). (B) Fluorescence measurements obtained by plate reader assay with both samples described in (A), where a clear fluorescence peak is observed when the GFPsensor grows (in this strain GFP is constitutively expressed). The maximum value of the fluorescence peak is considered as GFPsensor signal in all figures of this work and corrected by the correspondent optical density (OD_{600}), yielding the relative fluorescent values (RFU/OD_{600}). All values are corrected for background fluorescence.

A B

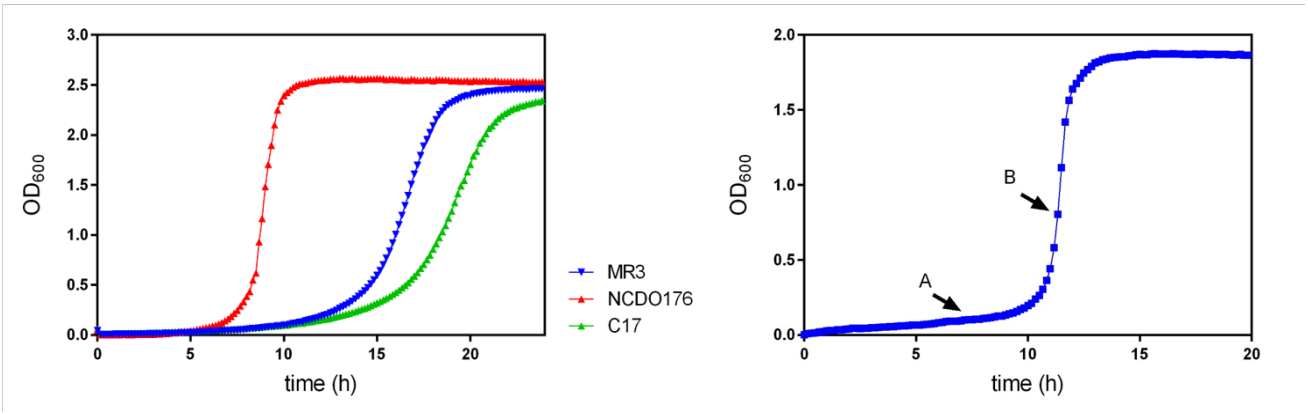

Fig. S5. The growth of some producer strains of the collection used for screening of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. (A) Although all the strains are inoculated with the same dilution and OD_{600} (see Methods), they differ in growth rate. This fact could be explained by different factors, some of them could be related to nitrogen metabolism such as differences in proteolysis rate, in the PrtP type or in the casein-derived peptide uptake systems. Importantly, this observation might explain the difference of time in the time-lapse experiments where the maximum growth of the GFPsensor strain is reported. (B) Collection of supernatant samples. Supernatant samples of the producer strains grown in CDM-casein were taken at two different growth points. The arrows indicate the two sampling points: A-end of lag-phase, corresponding to the time of the maximum GFP expression observed when co-cultivated with the GFPsensor, and B-mid exponential phase, as an indication of accumulated secreted amino acids, and to avoid amino acids released due to cell lysis that might occur at the stationary growth phase.

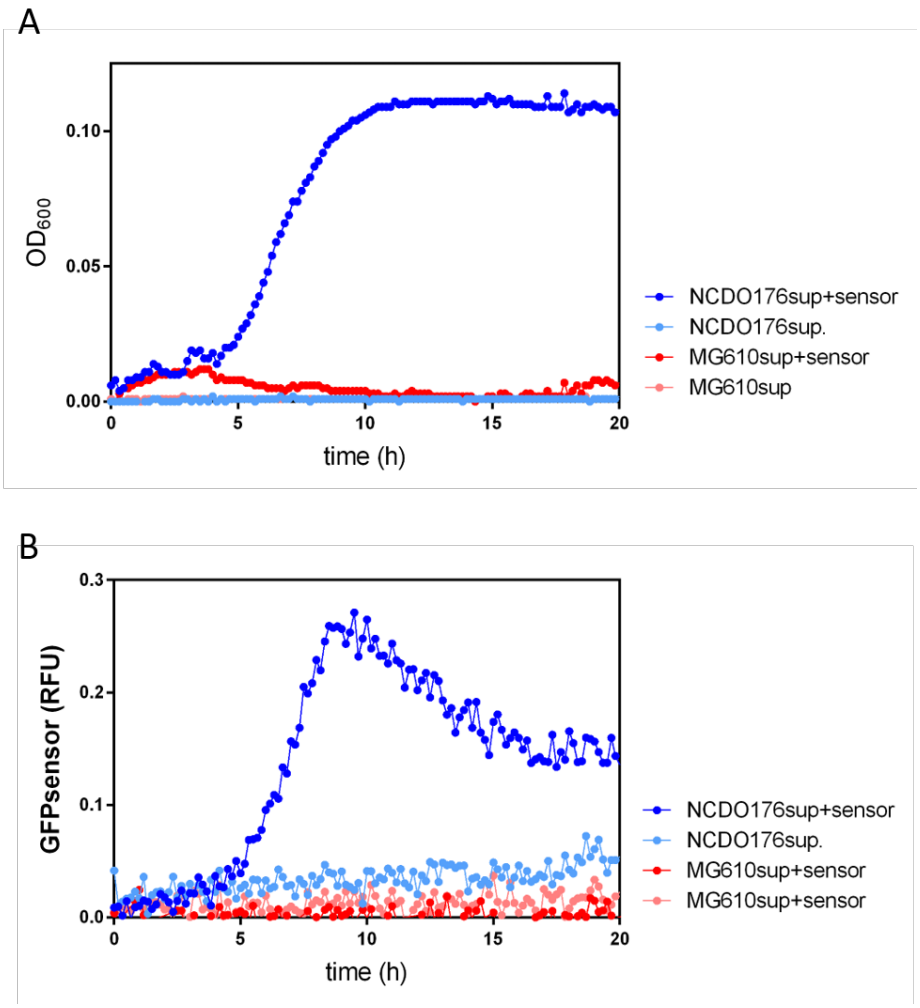

Fig. S6. The supernatant of the amino acid producers contain free essential amino acids. (A) Growth curve of the GFPsensor in the presence of supernatants of an amino acid producer (NCDO176, dark blue), and a non-producer (MG610, dark red). As controls, in light blue and light red lines, the supernatants without sensor cells. (B) GFP signal of the samples described in (A), shows that the GFPsensor is able to grow in the presence of only the supernatant of the amino acid producer (NCDO176).

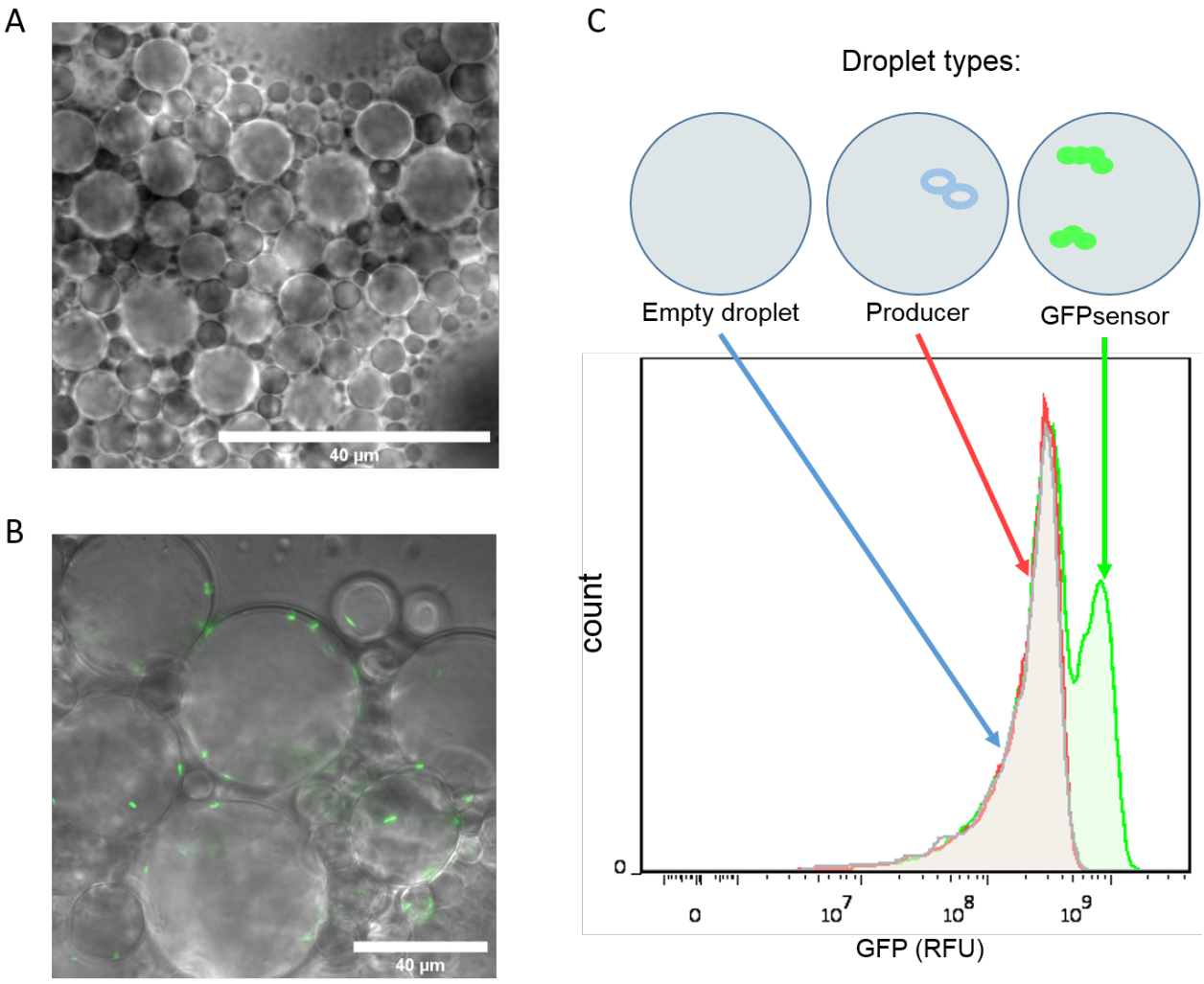

Fig. S7. Production of agarose-based droplets. (A) The high-performance dispersing instrument (Ultra Turrax type T25 Basic) creates droplets with a wide range of sizes. (B) GFPsensor cells are encapsulated in the droplets and observed with fluorescence microscopy. (C) In an experiment using CDM+aa (all amino acids present), the agarose-based droplets with grown cells GFPsensor are distinguished from empty droplets and droplets that contain only producer cells (GFP-).

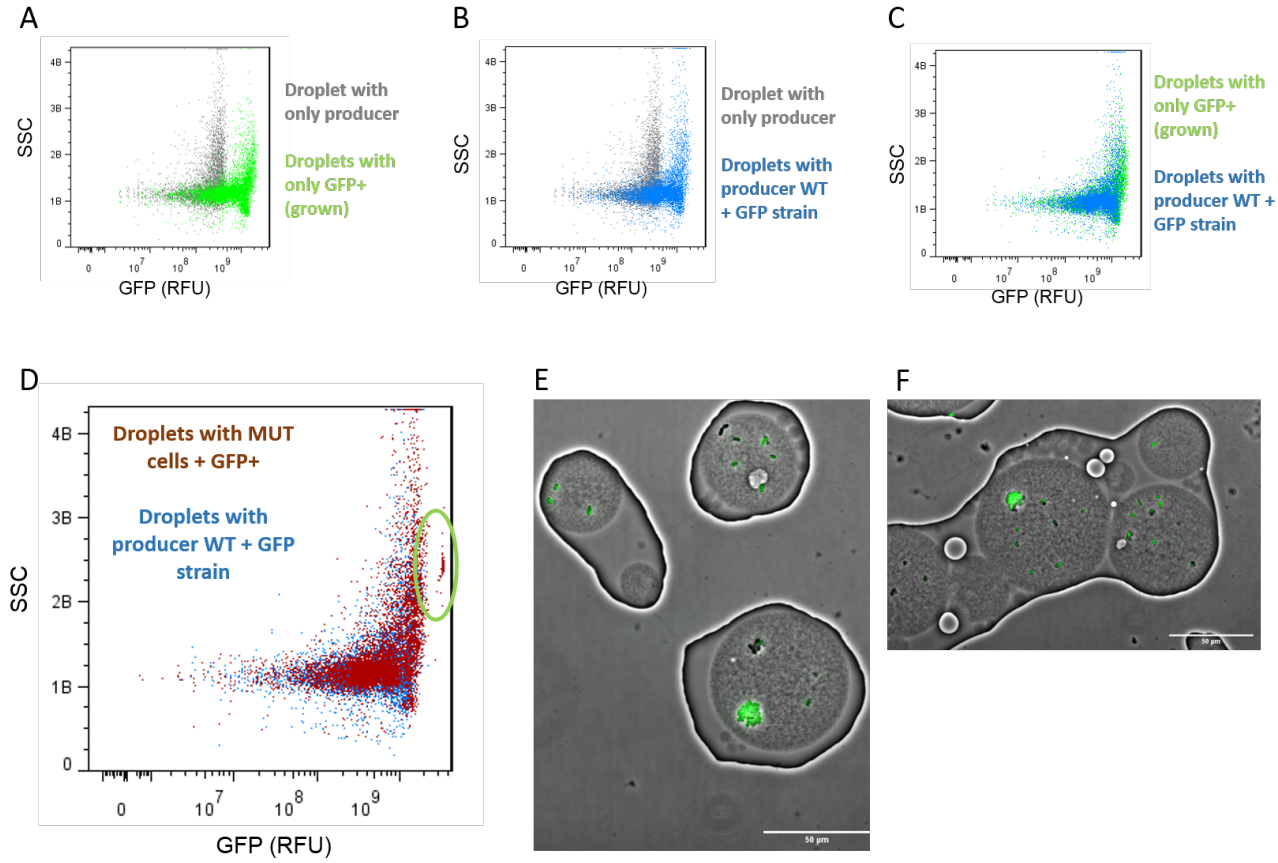

Fig. S8. Sorting of agarose-based droplets containing potential amino acid overproducer cells. (A) Droplets containing control samples, droplets with only GFPsensor cells grown in CDM+aa (green), and droplets with only producer cells (IPLA838 WT, in grey). (B) Droplets containing control samples, droplets with producer-GFPsensor (1:10 ratio) cells grown in CDM-casein (blue), and droplets with only producer cells (IPLA838 WT, in grey). (C) Droplets containing control samples, only GFPsensor cells grown in CDM+aa (green), and droplets with producer-GFPsensor (1:10 ratio) cells grown in CDM-casein (blue). (D) Droplets containing producer WT-GFPsensor (1:10 ratio) cells grown in CDM-casein (blue), and droplets with mutagenized producer-GFPsensor (1:10 ratio) cells grown in CDM-casein (dark red). The sorted droplets are indicated in a green circle. (E, F) Snapshots of droplets illustrating the different possible scenarios: empty droplets, droplets with only sensor, and droplets with producer-sensor.

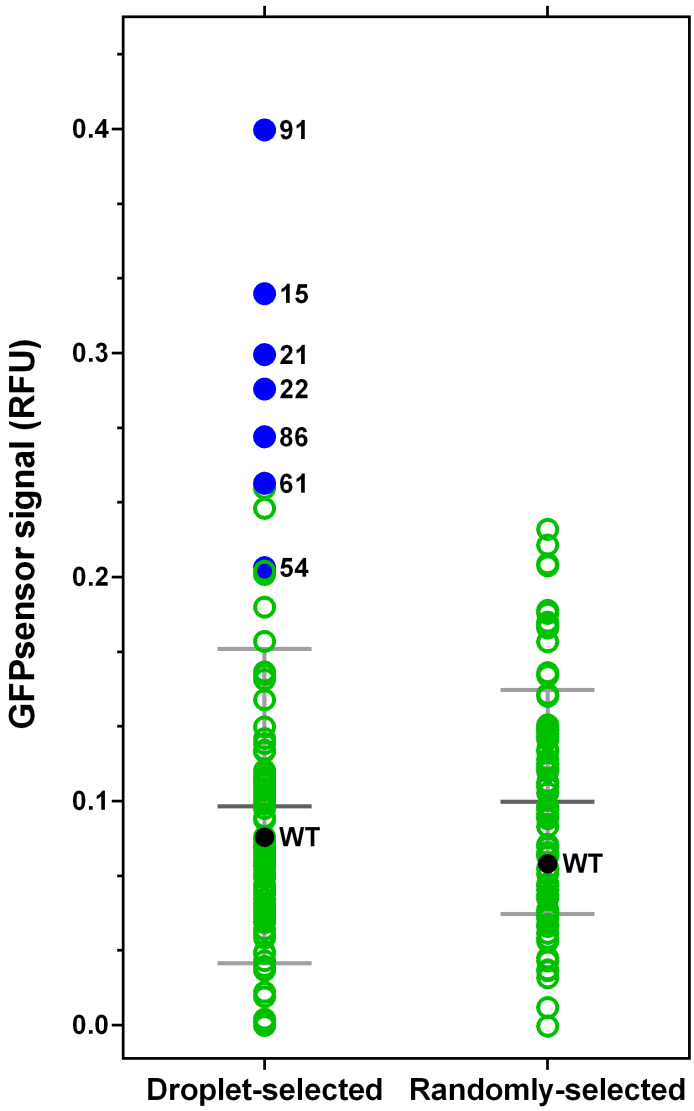

Fig. S9. Comparison between droplet- and randomly- selection screenings of overproducer strains. The sample in the left side of this plot shows the screening for amino acid secretion performed by droplet-selection (as shown in Fig. 3B) by 103 MUT strains by co-cultivation with the GFPsensor. The GFPsensor fluorescence signal (y-axis) when it is co-cultivated with each MUT strain is shown. The wild-type strain (WT) is indicated (black dot), and strains that highly promote the growth of the GFPsensor are highlighted (blue dots). The sample in the right side of this plot shows the screening of 100 randomly selected MUT strains, without droplet selection.

Table S1. Strains and plasmids used in this study

Strain	Description	Reference
L. lactis		
MG1363	Opp$^+$, DtpT$^+$, Dpp$^+$, Lac$^-$, Prt$^-$; *L. lactis* subsp. *cremoris,* plasmid-free NCDO712.	(Gasson, 1983)
AG500	Opp$^-$, DtpT$^-$, Dpp$^+$; MG1363 Δ*pepO*, Δ*dtpT*, Δ*opp.*	(Hagting et al., 1994; Kunji et al., 1995)
YS5A	Opp$^-$, DtpT$^-$, Dpp$^-$, Ery^r; AG500 *dppA*::pINT*dppA.*	(Sanz et al., 2001)
GFPsensor	Lac$^-$, Prt-, Ery^r, AG500 derivative, carrying the vector pSEUDO::P$_{usp45}$-*sfgfp(Bs).*	This work
NZ9000pLP712	Lac$^+$, Prt+, *L. lactis* subsp. *cremoris* NZ9000 carrying the conjugative plasmid of NCDO712 containing the *lac* and *prtP/prtM* genes.	MolGen collection
MG610	Ery^r, PrtP+, MG1363 derivative, carrying two to three copies of vector pKLG610 containing the *prtP/prtM* genes of *L. lactis* subsp. *cremoris* Wg2.	(Leenhouts et al., 1991)
MG1363pGKV552	Ery^r, Lac$^-$, PrtP+; MG1363 carrying the plasmid pGKV552 that contains the *prtP/prtM* genes of *L. lactis* subsp. *cremoris* Wg2.	(Leenhouts et al., 1991)
NCDO712	Lac$^+$, PrtP+, *L. lactis* subsp. *cremoris*	(Gasson, 1983)
SK11	Lac$^+$, PrtP+, *L. lactis* subsp. *cremoris*	(Siezen et al., 2005)
Wg2	PrtP+, *L. lactis* subsp. *cremoris*	(Haandrikman et al., 1990; Otto et al., 1982)
AM1	PrtP+, *L. lactis* subsp. *cremoris*	(Exterkate and De Veer, 1989)
RR2	Lac$^+$, PrtP+, *L. lactis* subsp. *lactis* biovar. diacetylactis	MolGen collection
WW4	Lac$^+$, PrtP$^+$, *L. lactis* subsp. *lactis* biovar. diacetylactis	MolGen collection
M18	Lac$^+$, PrtP+, *L. lactis* subsp. *lactis* biovar. diacetylactis	MolGen collection
CNRZ190	Lac$^+$, PrtP+, *L. lactis* subsp. *lactis* biovar. diacetylactis	(Obis et al., 2001)
1816S	Lac$^+$, PrtP+, *L. lactis* subsp. *lactis* biovar. diacetylactis	(Hill et al., 1985)

CRL264	Lac$^+$, PrtP+, *L. lactis* subsp. *lactis* biovar. diacetylactis	(García-Quintáns et al., 2008)
C17	Lac$^+$, PrtP+, *L. lactis* subsp. *lactis* biovar. diacetylactis	(Hugenholtz and Starrenburg, 1992)
NCDO176	Lac$^+$, PrtP+, *L. lactis* subsp. *lactis* biovar. diacetylactis	MolGen collection
IPLA838	Lac$^+$, PrtP+, *L. lactis* subsp. *lactis* biovar. diacetylactis	(Cárcoba et al., 2000)
MR3	Lac$^+$, PrtP+, *L. lactis* subsp. *lactis* biovar. diacetylactis	(Monnet et al., 2000)
E. coli		
DH5α	F$^-$ φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(rK$^-$, mK$^+$) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Laboratory stock

Plasmids	**Description**	**Reference**
pSEUDO::P$_{usp45}$-*sfgfp(Bs)*	Ery^r, integration vector, pSEUDO::P$_{usp45}$-*sfgfp(Bs)* derivative, carrying the gene coding for the green fluorescent protein (sfGFP).	(Overkamp et al., 2013)

Table S2. Oligonucleotides used in this study

Name	Sequence
TestPrtP_Fw1	5’ ACTTATAGTCCTGCTGGTGGTAATT 3’
TestPrtP_Rv1	5’ CTTCATTAATTGCAGTTTTAACGCTC 3’

Table S3. Identified mutations in the genomic DNA sequences of three MUT cells.

MUT cell	Mutation	Gene	Annotation
MUT-91	Q57L (CAA→CTA)	*IPLA838_01463*	Hypothetical protein
	N2K (AAC→AAA)	*oppA*	Oligopeptide-binding protein
	(TGT→A*T) (intergenic)	P*oppA*	Promoter Oligopeptide-binding protein
	V87L (GTT→CTT)	*IPLA838_02283*	Hypothetical protein
	F508S (TTT→TCT)	*IPLA838_00548*	peptidase
	E504G (GAA→GGA)	*IPLA838_00548*	peptidase
	C21Y (TGT→TAT)	*IPLA838_00550*	Hypothetical protein
	F28L (TTT→CTT)	*IPLA838_00937*	Hypothetical protein
	A22V (GCT→GTT)	*ftsA*	Cell division protein
	D14E (GAT→GAA)	*IPLA838_02003*	Hypothetical protein
	H266L (CAT→TAT)	*purL*	Phosphoribosyl-formyl-glycinamidine synthase subunit
	A51V (GCT→GTT)	*menH_1*	2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate synthase
	E155K (GAA→AAA)	*IPLA838_02454*	Hypothetical protein
	Q76* (CAA→TAA)	*IPLA838_00812*	Putative endopeptidase
MUT-15	Q57L (CAA→CTA)	*IPLA838_01463*	Hypothetical protein
	N2K (AAC→AAA)	*oppA*	Oligopeptide-binding protein
	(TGT→A*T) (intergenic)	P*oppA*	Promoter Oligopeptide-binding protein
	V87L (GTT→CTT)	*IPLA838_02283*	Hypothetical protein
	F508S (TTT→TCT)	*IPLA838_00548*	peptidase
	E504G (GAA→GGA)	*IPLA838_00548*	peptidase
	C21Y (TGT→TAT)	*IPLA838_00550*	Hypothetical protein
	F28L (TTT→CTT)	*IPLA838_00937*	Hypothetical protein
MUT-21	Q57L (CAA→CTA)	*IPLA838_01463*	Hypothetical protein
	N2K (AAC→AAA)	*oppA*	Oligopeptide-binding protein
	(TGT→A*T) (intergenic)	P*oppA*	Promoter Oligopeptide-binding protein
	V87L (GTT→CTT)	*IPLA838_02283*	Hypothetical protein
	F508S (TTT→TCT)	*IPLA838_00548*	peptidase
	E504G (GAA→GGA)	*IPLA838_00548*	peptidase
	C21Y (TGT→TAT)	*IPLA838_00550*	Hypothetical protein
	F28L (TTT→CTT)	*IPLA838_00937*	Hypothetical protein

The mutations highlighted in light orange color are shown in Fig. 5 as common mutations between the *L. lactis* MUT strains. These mutations are related to the lactococcal peptide uptake systems.

Table S4. Determination of proteinase type based on the amino acid residues of the PrtP protein.

PrtP residue	NCDO176	WW4	CRL264	SK11	Wg2
131	Thr	Thr	Thr	Thr	Thr
138	Thr	Thr	Thr	Thr	Thr
142	Ala	Ala	Ala	Ala	Ser
144	Leu	Leu	Leu	Leu	Leu
166	Asp	Asp	Asp	Asp	Asp
177	Leu	Ile	Ile	Leu	Leu
747	Leu	Leu	Leu	Arg	Leu
748	Thr	Thr	Thr	Lys	Thr
763	His	His	His	Asn	Asn
PrtP type	**f (PI/PIII)**	**f (PI/PIII)**	**f (PI/PIII)**	**b (PIII)**	**e (PI)**

Table S5. Proteinase (PrtP) types of positive- and negative-amino acid producing strains.

Strain	Subspecies	PrtP type	Amino acid secretion
Wg2	*cremoris*	I	+
WW4	*lactis*	I/III	+
NCDO176	*lactis*	I/III	+
MG1363pNZ521	*cremoris*	III	-
MG1363pGKV552	*cremoris*	I	-
CRL264	*lactis*	I/III	-
SK11	*cremoris*	III	-

Table S6. Contents of essential amino acids in culture supernatants of MUT strains.

Sample		Glu	His	Val	Met	Ile	Leu
CDM-casein	B	ND	ND	ND	ND	ND	ND
WT	A	1.3 ± 0.2	3.0 ± 0.2	ND	ND	ND	ND
	B	4.4 ± 0.1	3.0 ± 0.2	2.3 ± 0.1	ND	ND	ND
91	A	21.6 ± 0.7	5.9 ± 0.7	7.3 ± 0.4	ND	ND	7.5 ± 0.1
	B	47.5 ± 0.7	6.5 ± 0.4	23.8 ± 0.1	ND	2.5 ± 0.1	30.6 ± 0.7
15	A	19.1 ± 0.2	5.4 ± 0.3	4.6 ± 0.2	ND	ND	3.4 ± 0.2
	B	49.8 ± 0.3	6.8 ± 0.2	21.6 ± 0.2	ND	2.1 ± 0.1	21.6 ± 0.1
21	A	17.8 ± 0.3	6.1 ± 0.3	3.4 ± 0.4	ND	ND	3.9 ± 0.3
	B	38.6 ± 0.8	6.8 ± 0.3	18.3 ± 0.4	ND	ND	21.4 ± 0.7
22	A	14.3 ± 0.3	5.9 ± 0.2	1.4 ± 0.1	ND	ND	2.6 ± 0.4
	B	30.2 ± 0.3	7.0 ± 0.3	13.1 ± 0.2	ND	ND	14.7 ± 0.2
86	A	17.3 ± 0.2	6.0 ± 0.1	3.4 ± 0.2	ND	ND	4.1 ± 0.6
	B	42.7 ± 0.7	6.6 ± 0.2	19.1 ± 0.4	ND	1.9 ± 0.6	23.1 ± 0.2
61	A	13.3 ± 0.1	5.6 ± 0.2	1.3 ± 0.2	ND	ND	2.5 ± 0.1
	B	36.1 ± 0.2	6.1 ± 0.3	14.4 ± 0.3	ND	1.4 ± 0.2	16.6 ± 0.4
54	A	14.5 ± 0.2	6.1 ± 0.2	2.8 ± 0.2	1.3 ± 0.2	ND	2.6 ± 0.1
	B	33.8 ± 0.3	5.8 ± 0.2	16.0 ± 2.2	1.4 ± 0.1	1.2 ± 0.1	16.1 ± 0.1

Values indicate concentration (μM). Two sampling points are shown: A- end of lag growth phase, and B- mid exponential growth phase (see Fig. S4). After the strains were grown in CDM-casein medium, the concentrations of amino acids in the culture supernatants were measured using a HPLC assay coupled with fluorescence detection. The wildtype strain is shown in grey. All mutants are shown in blue. A negative (CDM-casein) control is shown in light grey. Amino acids below the detection limit of the HPLC assay are indicated as ND. Quantifications were performed in duplicates, and average values are shown. Error is shown as SD.

Supplementary References

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