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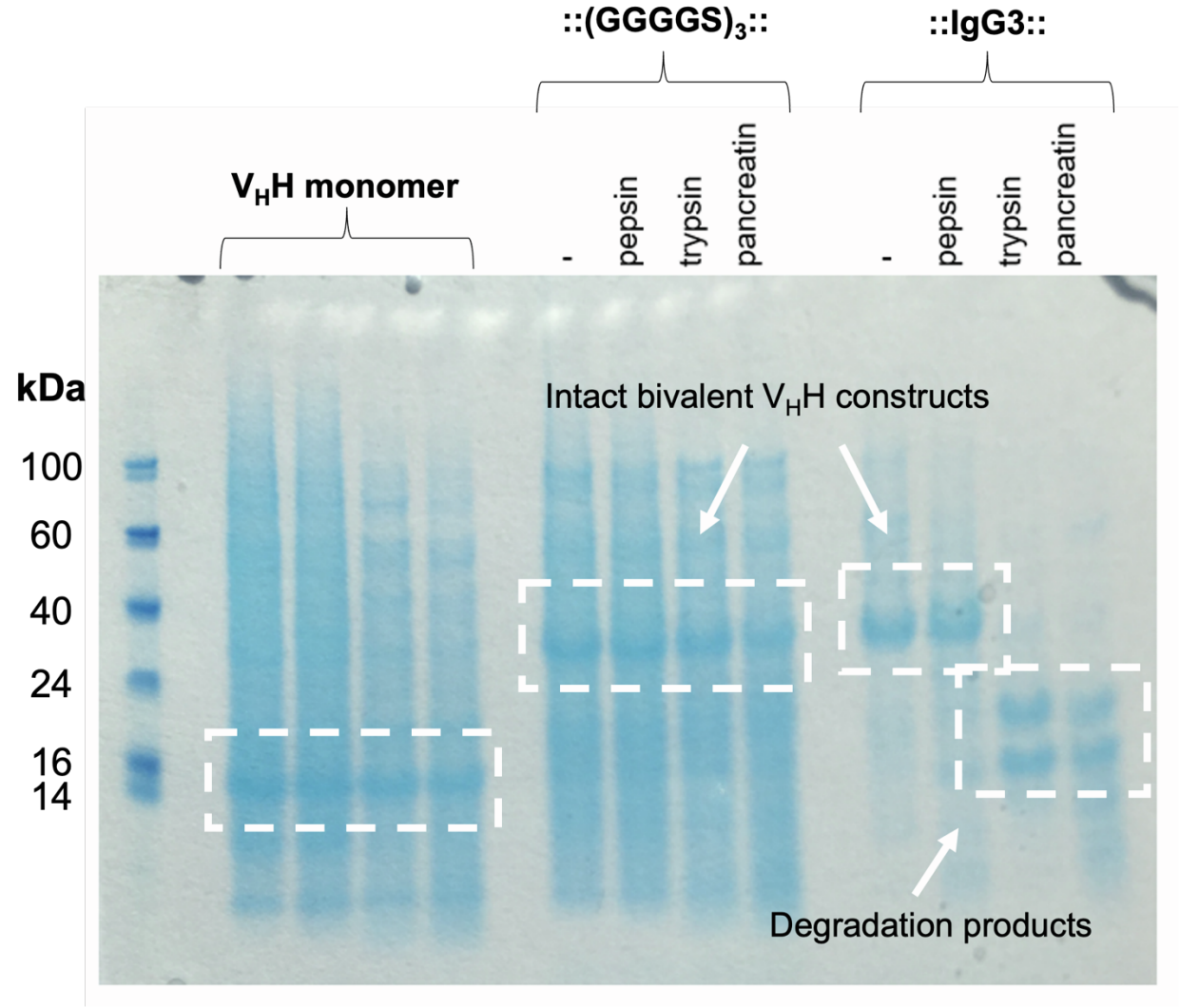
Supplemental information

Orally active bivalent V_HH construct prevents proliferation of $F4^+$ enterotoxigenic *Escherichia coli* in weaned piglets

Berthe Katrine Fiil, Sandra Wingaard Thrane, Michael Pichler, Tiia Kittilä, Line Ledsgaard, Shirin Ahmadi, Grith Miriam Maigaard Hermansen, Lars Jelsbak, Charlotte Lauridsen, Susanne Brix, and Andreas Hougaard Laustsen

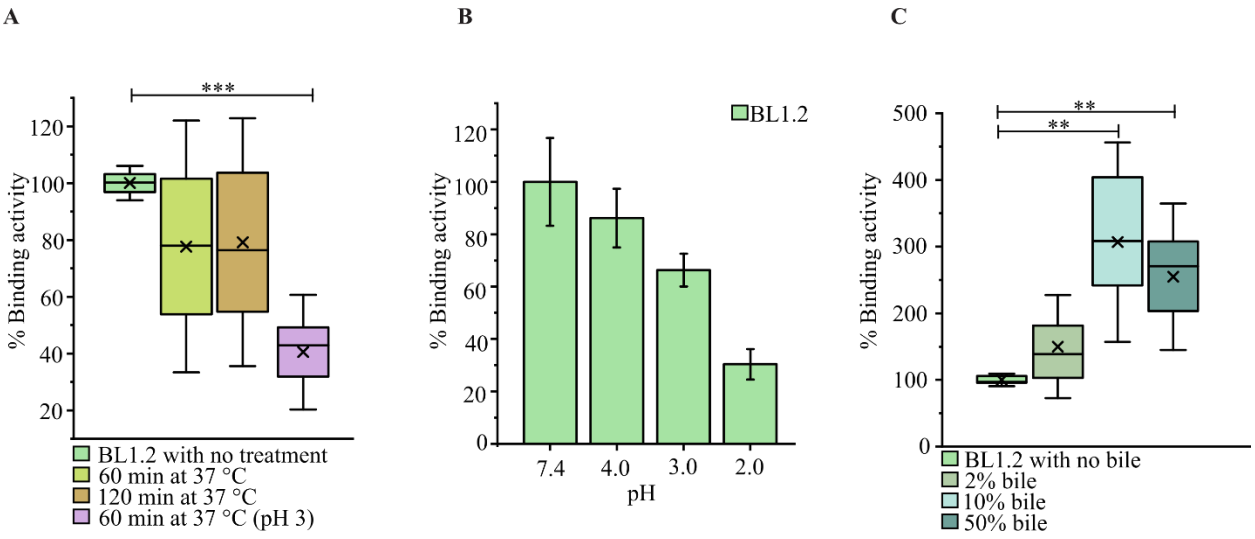
Supplementary Information

Fig. S1.



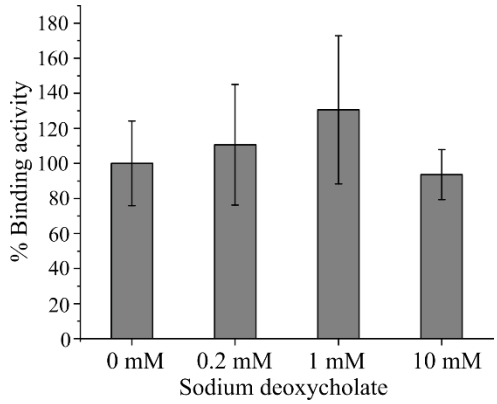
Supplementary Figure S1. The bivalent V_HH construct comprising a glycine-serine linker shows increased proteolytic stability compared to a similar bivalent V_HH construct comprising an IgG3 linker. Related to Figure 1. After incubation with pepsin at pH 3, trypsin at pH 8, or pancreatin at pH 8, the bivalent V_HH construct based on a glycine-serine linker (::GGGGS3::) remained stable, while the bivalent V_HH construct based on a IgG3 linker (::IgG3::) was degraded to monomers after incubation with trypsin or pancreatin.

Fig. S2.



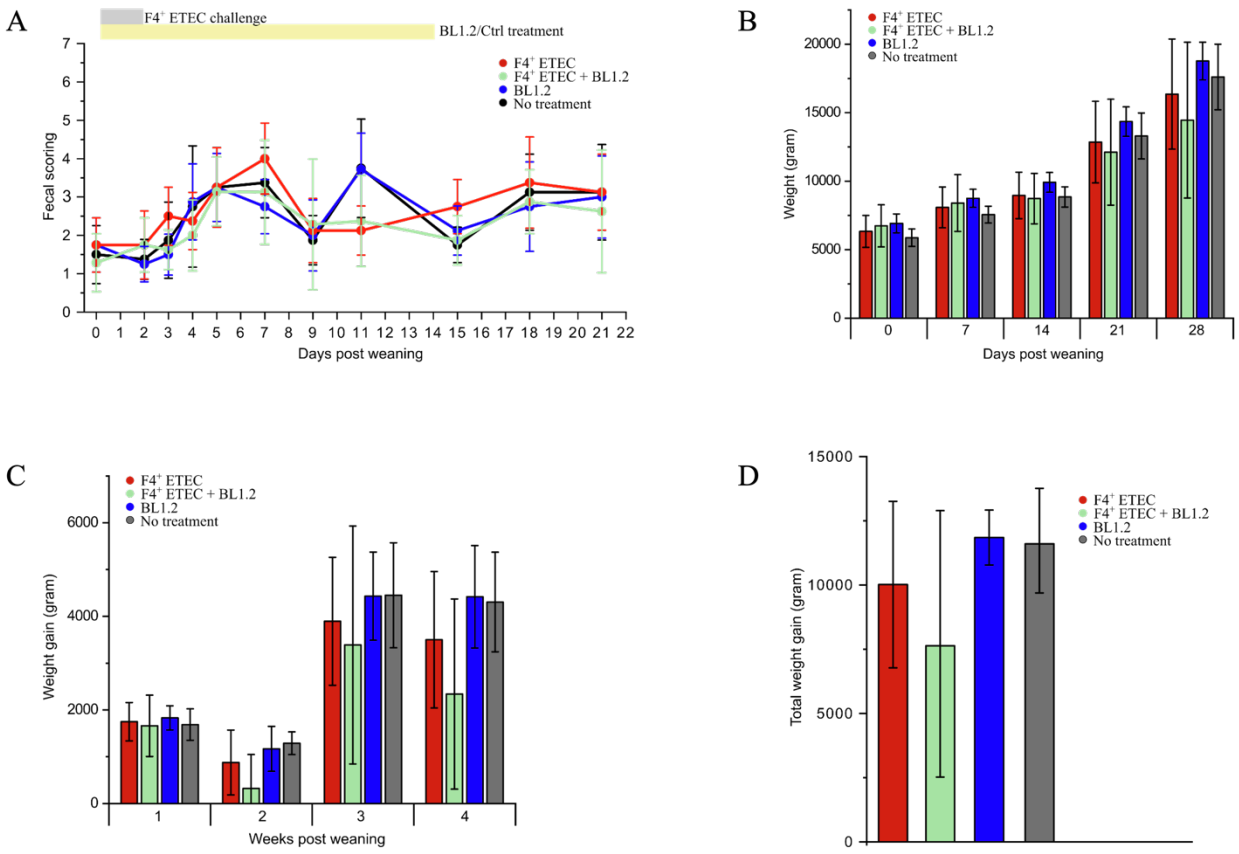
Supplementary Figure S2. The bivalent BL1.2 V_HH construct preserves stable binding to F4$^+$ ETEC upon exposure to different conditions present in the pig intestine. Related to Figure 2. A) Remaining binding activity of the bivalent BL1.2 V_HH construct to F4$^+$ ETEC upon pre-incubation in freshly collected pig gastric juice for up to two hours. B) Remaining binding activity of BL1.2 to F4$^+$ ETEC after pre-incubation at different pH (adjusted with HCl to mimic pig gastric acid) for 1 h at 37°C. Data represents mean ± SD. C) Binding of BL1.2 to F4$^+$ ETEC in the presence of freshly collected pig bile at different concentrations. The level of BL1.2 binding to F4+ ETEC (A-C) was determined indirectly; product (pretreated or not) was used to coat the wells at a concentration within the dynamic range of the assay, F4$^+$ ETEC was added to saturating levels, and BL1.1-FLAG was used to determine the level of bound F4$^+$ ETEC. ELISA-based binding analyses (A-C) were performed in at least three independent biological replicates ($n \geq 3$). For binding activities presented in A and C, the median is shown as a line, mean with an X, and whiskers show the standard deviation (SD). An one-way Anova was used to determine statistical significance between the individual groups and a Tukey's range test was applied *post hoc* to identify significant different groups; Tukey's test: *, $p < 0.05$ **, $p < 0.01$; ***, $p < 0.001$.

Fig. S3.



Supplementary Figure S3. Effects of sodium deoxycholate on binding of the BL1.2 construct to F4$^+$ ETEC. Related to Figure 2. Binding of BL1.2 to F4$^+$ ETEC in the presence of sodium deoxycholate at different concentrations. The level of BL1.2 binding to F4$^+$ ETEC was determined by indirect ELISA, where BL1.2 was used to coat the wells, F4$^+$ ETEC was added to saturating levels in the presence of sodium deoxycholate, and BL1.1-FLAG was used to determine the level of bound F4$^+$ ETEC. Binding assays were performed as independent biological triplicates and data represent mean ± SD.

Fig. S4.



Supplementary Figure S4. Data on effect of F4+ ETEC infection in piglets. Related to Figure 4. A) Fecal scoring of piglets in individual treatment groups over a periode of 21 days post weaning (Bristol stool scale: 1 = hard, dry and cloddy, 2 = firm, 3 = soft with shape, 4 = soft and liquid, 5 = watery and dark, 6 = watery and yellow, 7 = foamy and yellow). B) Body weight of piglets in individual treatment groups over a periode of 28 days post-weaning. C) Weekly weight gain of piglets in individual treatment groups over a periode of 4 weeks post-weaning. D) Total weight gain of piglets in individual treatment groups over a periode of 28 days post-weaning. (A-D) Piglets in treatment groups (n = 8) "F4+ ETEC" and "F4+ ETEC + BL1.2" were challenged by gavage with 1-1.7 x 10^9 F4+ ETEC at day one and two post-weaning. The non-challenged groups were gavaged with sodium bicarbonate. BL1.2 or the control protein, chicken egg albumin (Ctrl), which were administered to the "F4+ ETEC" and "no treatment" groups, respectively, were provided from day 1 to 14 via oral gavage. (A-D) Data represents mean ± SD. (A-D) Presented data from the individual treatment groups are not significantly different (ANOVA, $p < 0.05$).

Supplementary Table S1. Genome analysis of the AUF4 and AUF18 porcine ETEC strains. Related to STAR Methods.

Name	Genome size (bp)	CDS	Contigs	N50	%GC	Serogroup	MLST	Adhesins	Enterotoxins
AUF4	5,370,933	5,211	220	113,087	50.62	O149:H10	ST100	*faeG, csgG, eaeH, ecpA*	*east1, eltB, estB*
AUF18	5,576,275	5,420	277	101,135	50.42	O138:H14	ST42	*fedF, fimH, csgG, eaeH, sfmA, ecpA*	*east1, stx2e, hlyE, eltB, estB*

Supplementary Table S2. Mass spectrometry-based identification of BL1.1 from the acid eluate. Related to Figure 1. Table summarizes identified peptides of BL1.1 and search engine retrieved quality scores thereof. Identification was based on a coverage of 85%, 129 reads, and 14 unique peptides.

Conf	Sequence	Modifications	PSMs	Master Protein Accessions	Missed cleavages	Theo. MH+ [Da]	Confidence (by Search Engine): Sequest HT	XCorr (by Search Engine): Sequest HT
High	DNAKNTVYLQMNSLKPEDTAVYYCAAGR	1xCarbamidomethyl [C24]	1	BL1.1	1	3192.50886	High	7.52
High	NTVYLQMNSLKPEDTAVYYCAAGR	1xCarbamidomethyl [C20]; 1xOxidation [M7]	10	BL1.1	0	2780.30183	High	6.27
High	NTVYLQMNSLKPEDTAVYYCAAGR	1xCarbamidomethyl [C20]	6	BL1.1	0	2764.30691	High	7.54
High	GRFTISRDNAK		8	BL1.1	2	1264.67566	High	3.57
High	GRFTISR		1	BL1.1	1	836.47371	High	2.13
High	GGGSSYYADSVKGRFTISR		3	BL1.1	2	2007.98828	High	3.9
High	GGGSSYYADSVKGR		3	BL1.1	1	1403.65498	High	4.41
High	GGGSSYYADSVK		4	BL1.1	0	1190.53241	High	3.36
High	GAPSDTGRPDEYDYWGQGTQVTVSS		15	BL1.1	0	2673.16994	High	6.56
High	FTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGR	1xCarbamidomethyl [C29] 1xOxidation [M16]	2	BL1.1	2	3812.83707	High	4.39
High	FTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGR	1xCarbamidomethyl [C29]	4	BL1.1	2	3796.84215	High	7.89
High	FTISRDNAK		2	BL1.1	1	1051.55308	High	2.59
High	EREFVATVSRGGGSSYYADSVK		21	BL1.1	2	2365.14188	High	6.07
High	EREFVATVSR		6	BL1.1	1	1193.62731	High	3.67
High	EFVATVSRGGGSSYYADSVKGR		3	BL1.1	2	2293.12075	High	4.42
High	EFVATVSRGGGSSYYADSVK		5	BL1.1	1	2079.99817	High	4.57
High	EFVATVSR		2	BL1.1	0	908.48361	High	2.09
High	QAPGKEREFVATVSR		5	BL1.1	2	1674.89219	High	3.83
High	QVQLQESGGGLVQPGGSLR		28	BL1.1	0	1910.00901	High	5.6

PSM: peptide-spectrum match. **Theo. MH+**: Theoretical mass of protonated peptides.

Supplementary Table S3: Source data of $F4^+$ ETEC challenge in post-weaning piglets. Related to Figure 4.

Treatment group	Number of piglets	$F4^+$ ETEC challenge	BL1.2 administration	Time point in day (D) / Number of infected piglets										
				D0	D2	D3	D4	D5	D7	D9	D11	D15	D18	D21
$F4^+$ ETEC	8	Yes	No	0	4*	5	6*	7	8	6*	3***	1	0	2
$F4^+$ ETEC + BL1.2	8	Yes	Yes	0	2*	5	6**	2*	1	1*	0	2*	0	3
BL1.2	8	No	Yes	0	0	0	0	0	0	0	0	0	0	0
No treatment	8	No	No	0	0	0	0	0	0	0	0	0	0	0

*) 1 pig with one positive colony. **) 2 pigs with 2 positive colonies; 1 pig with 1 positive colony.***) 2 pigs with 1 positive colony; 1 pig with 2 positive colonies. When no asterisks are present, 5/5 colonies tested per pig were positive.

Supplementary Table 4. Summary of blood sample analysis from F4+ ETEC challenged post-weaning piglets. Related to Figure 4.

	BL2.1		Control	
	No $F4^+$ ETEC	$F4^+$ ETEC	No $F4^+$ ETEC	$F4^+$ ETEC
Red blood cells (10^{12}/L)	6.58	6.45	6.14	6.53
Hematocrit (%)	39.2	38.2	38.2	38.2
Hemoglobin (mmol/L)	11.7	11.2	11.6	11.6
Leukocytes (10^9/L)	14.0	14.9	14.5	14.0
Lymphocytes (%)	54.4	60.7	57.1	52.5
Neutrophils (%)	39.8	32.8	37.3	41.9
Monocytes (%)	4.58	5.25	4.39	4.20

Data are means of 8 piglets per groups.

Supplementary Table 5. Summary of previously described ETEC adhesin and toxin genes, which were used in this study to verify and outline virulence gene profiles of the *de novo* assembly of AUF4 and AUF18 via BLASTn. Related to STAR Methods.

	Gene	Accession no.	Gene product	Size (bp)
Adhesins	*faeG*	M29375	F4 fimbrial tip adhesin	840
	fedF	AY970782	F18 fimbrial tip adhesin	918
	fimH	X05672	Type 1 fimbriae, D-mannose-specific adhesin	2050
	csgG	EU902647	Facilitator of fibronectin-binding curli assembly	834
	eaeH	DQ109813	Highly conserved adhesin	4257
	sfmA	ASI53589	Fimbrial-like adhesin	573
	ecpA	ACI29343	*E. coli* common pilus	588
Enterotoxins	*east1*	AB042002	Enteroaggregative *E. coli* heat-stable enterotoxin (EAST1)	117
	stx2e	AJ313016	Shiga like toxin type 2e (stx2e)	1509
	hlyE	AB646137	Hemolysin E (HlyE)	1254
	eltB	M17873	Heat-labile enterotoxin B subunit (LT)	604
	estA	M58746	Heat-stable enterotoxin a (STa)	667
	estB	P22542	Heat-stable enterotoxin b (STb)	216