Human glucose-dependent insulinotropic polypeptide (GIP) is an antimicrobial adjuvant re-sensitising multidrug-resistant Gram-negative bacteria

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Supplementary material

Contents

Bacteria

Wild type *E. coli TG1* and *E. amylovora* and their *acrAB-tolC* mutations were sourced as follows. *E. coli TG1 subE hsd*Δsd*thi* Δ(*lac-proAB*) F`[*traD*36 *proAB*$^{+}$ *lacI*^{q} *lacZ* ΔM15] (Sambrook and Russel, 2001); KAM3-1, Gm^{r}, *tolC* mutant of KAM3 (*acrB* mutant of TG1) (Al-Karablieh et al., 2009a); *E. amylovora* 1189 wild type (GSPB, Göttinger Sammlung phytopathogener Bakterien, Göttingen, Germany); *E. amylovora* 1189-3-3 Km^{r}, Gm^{r}, *acrB tolC* mutant of *E. amylovora* 1189 (Al-Karablieh et al., 2009b).

General procedures

Solid-phase peptide synthesis employed 9-fluorenylmethoxycarbonyl chemistry (Fmoc-SPPS) (Jaradat, 2018, Li *et al.*, 2019). All Fmoc-amino acids, solvents, resins and reagents, were obtained from commercial suppliers and used without further purification. An APPTEC Focus Xi synthesizer was used. A Shimadzu LC-10AT vp analytical high-performance liquid chromatography (HPLC) system at 220 nm, was used to record HPLC spectra using a Phenomenex C18 column (150 x 4.6 mm, 5 μm). An Agilent 1200 – API 4000 LC–MS/MS system was used for recording mass spectra. A preparative Knauer HPLC system was used for peptide purification using a Phenomenex C18 column (250 x 21.2 mm,10 μm) (Jaradat *et al.*, 2019).

Synthesis and characterization of human GIP (peptide 3)

Human GIP Fmoc-SPPS was achieved using the pseudoproline dipeptides (Haack and Mutter, 1992, Wöhr and Mutter, 1995) Fmoc-Tyr(tBu)-Ser($\psi^{Me,Me}$Pro)-OH and Fmoc-Ile-Thr($\psi^{Me,Me}$Pro)-OH in order to disrupt any potential aggregation encountered with the synthesis of the peptide during synthesis. Pre-loaded resin (Scheme **S1**), Fmoc-Gln(Trt)-Wang resin (0.2632 g, 0.1 mmol, 0.38 mmol/gram, 1 equiv), was swollen in dimethylformamide (DMF) for about one hour, next it

was treated with 20% piperidine in DMF (3 × five minutes) followed by washing with DMF (3 × one minute), CH_2Cl_2 (3 × one minute) and finally with DMF (3 × one minute). Elongation of peptide chain was achieved by incorporating Fmoc-protected amino acids (5 equiv) together with standard amide coupling conditions 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) amide coupling conditions. Pseudoproline dipeptides Fmoc-Tyr(tBu)-Ser($\psi^{Me,Me}$Pro)-OH and Fmoc-Ile-Thr($\psi^{Me,Me}$Pro) were incorporated in residues 10-11 and 40-41, respectively. Next, peptidyl-resin (**2**) was treated with 20% piperidine in DMF, washed with DMF then dichloromethane (DCM) and dried. After peptide assembly, it was treated with trifluoroacetic acid (TFA):thioanisole:ethanediol:water (90:5:2.5:2.5 v/v, a total volume of 22 mL/g resin) for 4 hours to cleave the peptide from the resin and remove all side-chain protecting groups. The crude peptide was then precipitated in cold diethylether, dissolved in methanol/water and charged onto a preparative HPLC column. After freeze drying, purified human GIP was characterized and analyzed by LC–MS/MS and HPLC (Figure S1). LC–MS/MS (ESI): m/z = 713.00 g/mol $[M+7H]^{7+}$ (calcd.: m/z = 712.93 g/mol), 831.65 g/mol $[M+6H]^{6+}$ (calcd.: m/z = 831.59 g/mol), 997.70 g/mol $[M+5H]^{5+}$ (calcd.: m/z = 997.70 g/mol), 1246.85 g/mol $[M+4H]^{4+}$ (calcd.: m/z = 1246.87 g/mol), 1662.15 g/mol $[M+3H]^{3+}$ (calcd.: m/z = 1662.17 g/mol). Peptide **3** (human GIP) eluted at 14.3 min (flow rate: 0.1 min at 21 % CH_3CN (with 0.1 % TFA), gradient: 21 % to 40 % CH_3CN (with 0.1 % TFA) over 25 min then a gradient to 100 % CH_3CN (with 0.1 % TFA) over 0.1 min, then flow over 5 min at 100 % CH_3CN (with 0.1 % TFA) (Jaradat *et al.*, 2010).

The synthetic human GIP used in the studies by the Australian groups was purchased from GL Biochem (Shanghai, China).

Scheme S1

Solid-phase peptide synthesis of Human GIP incorporating pseudoproline dipeptides Fmoc-Tyr(tBu)-Ser($\psi^{Me,Me}$Pro)-OH and Fmoc-Ile-Thr($\psi^{Me,Me}$Pro) in residues 10-11 and 40-41, respectively. Fmoc = 9-fluorenylmethoxycarbonyl, Trt = trityl, TFA = trifluoroacetic acid, Fmoc-Xaa-OH = Fmoc-protected amino acids, PG = side chain protecting groups, SPPS = solid-phase peptide synthesis.

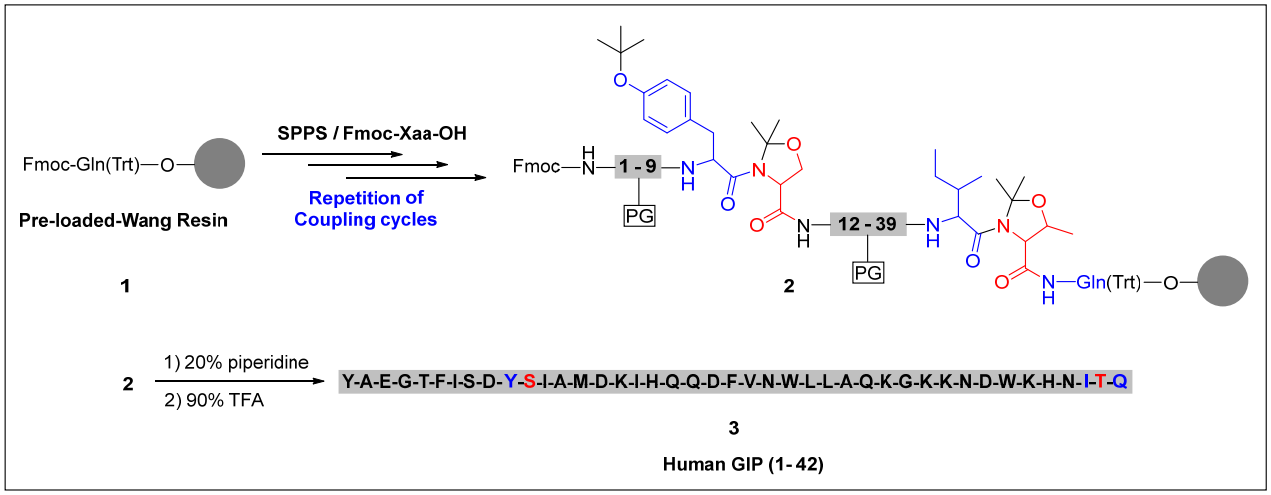

Figure S1. HPLC profile of synthetic human GIP (peptide 3).

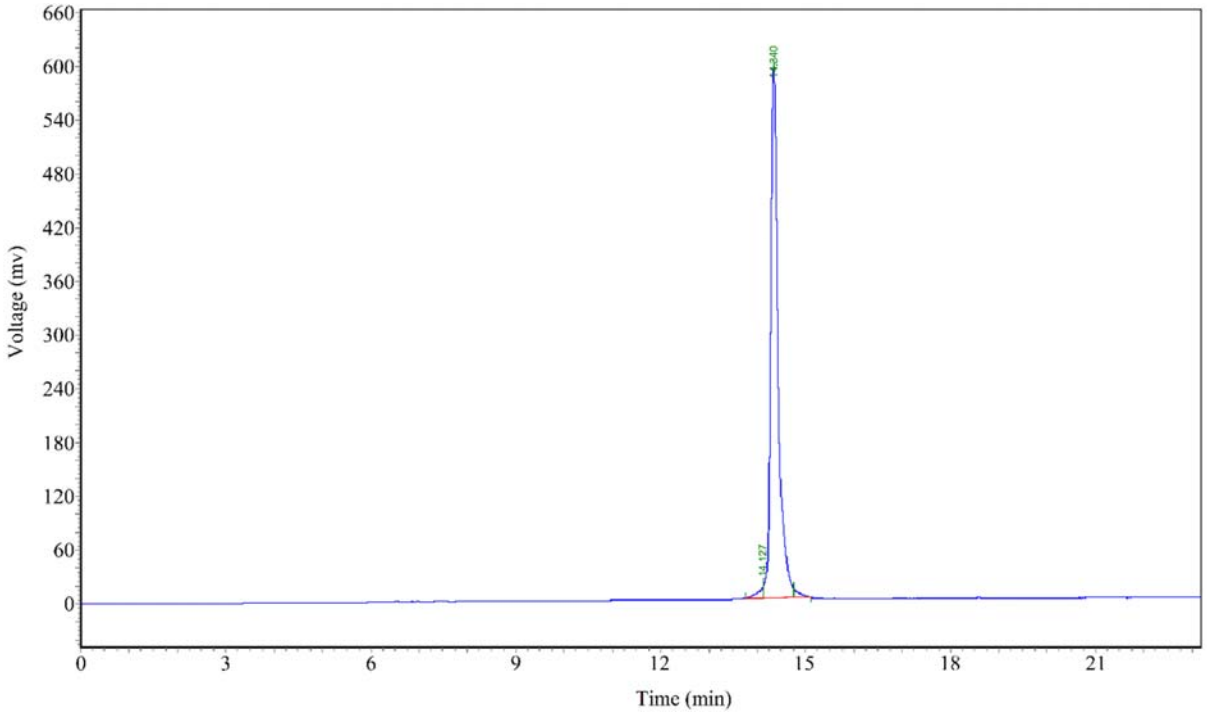

Figure S2. Flow cytometric dot plots of the membrane potential of E. coli ATCC 25922 after incubation with serial dilutions of GIP. The data were analysed in 3 biological replicates.

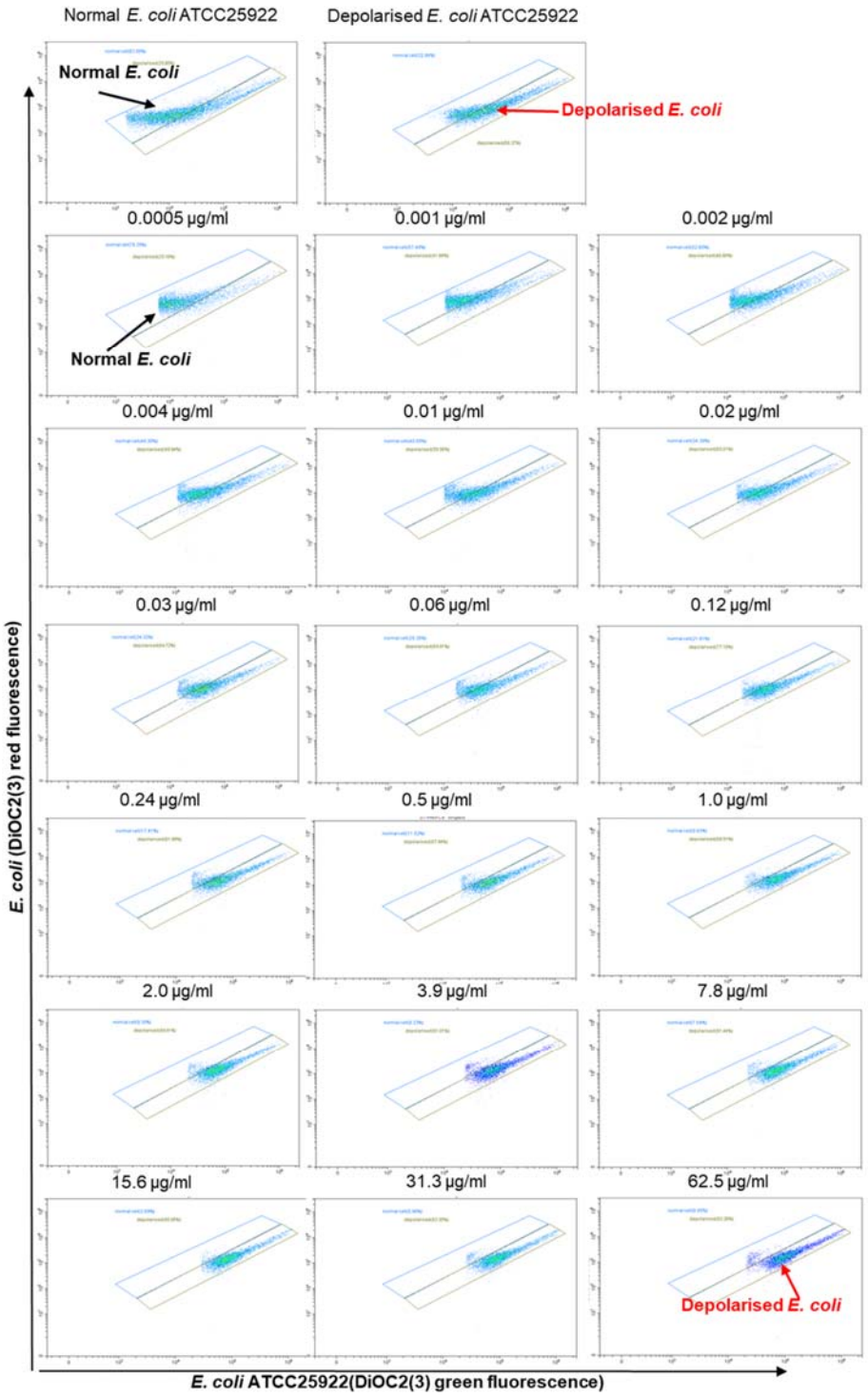

Figure S3. Induction of membrane permeability of E. coli ATCC 25922 by GIP as analysed by flow cytometry. The data were analysed in 3 biological replicates.

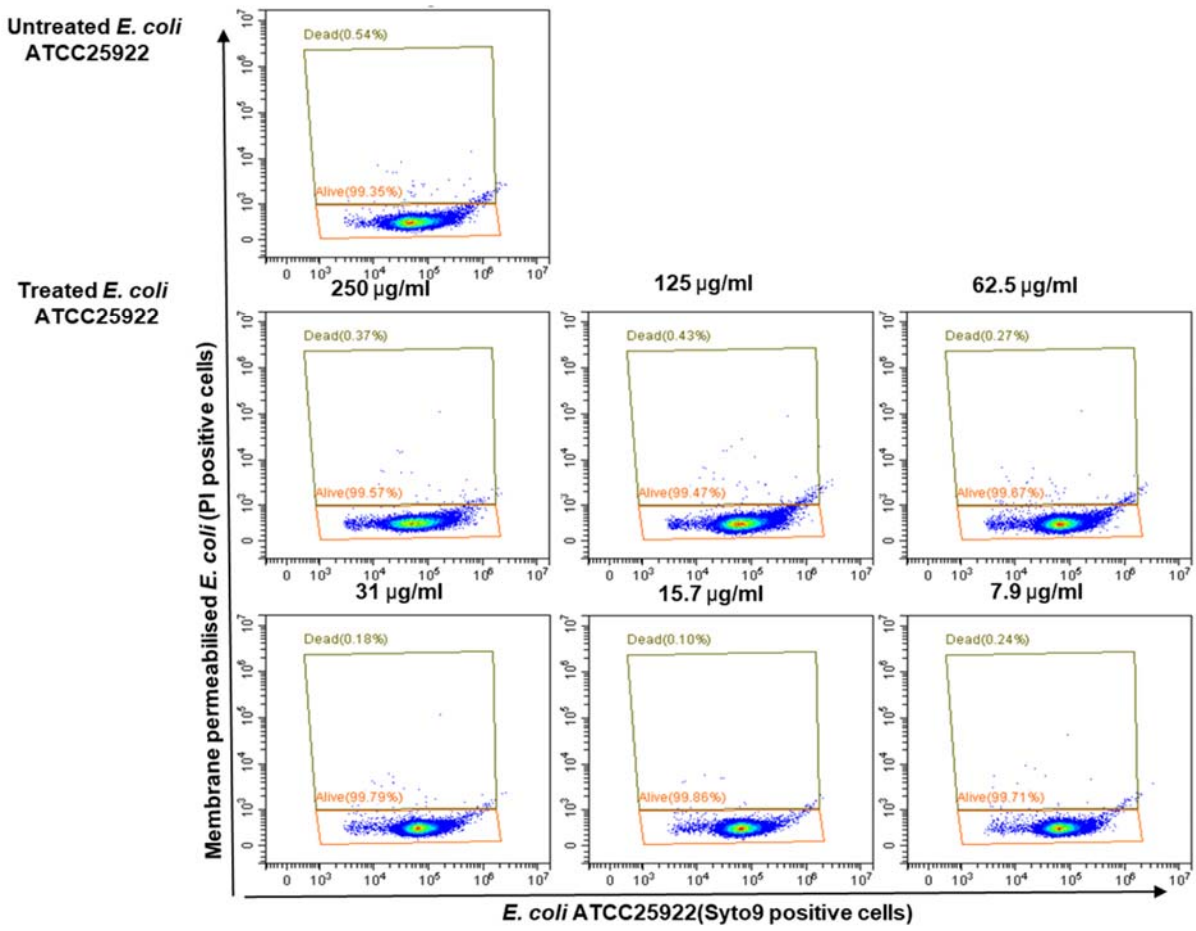

Table S1. Intercellular EtBr accumulation in *E. coli TG1* with human GIP. Relative fluorescence intensities at excitation and emission filters of 528/2 and 590/2 nm, respectively, over a period (up to 75 minutes). Average of four independent replicates ± relative standard deviation.

Time (min)	**PBS**	**EtBr Background**	***E. c* TG1 EtBr**	**E. c Δ*acrB*-Δ*tolC* EtBr**	***E. c* TG1 CCCP 12.5µg/mL**	***E. c* TG1 GIP 12.5µg/mL**	***E. c* TG1 GIP 25µg/mL**	***E. c* TG1 GIP 50µg/mL**	***E. c* TG1 GIP 100µg/mL**
0	0.5 ± 0.2	1.5 ± 0.33	7.25 ± 0.11	11.25 ± 0.04	8 ± 0.08	10 ± 0.07	9.25 ± 0.08	10.25 ± 0.07	9.75 ± 0.8
15	0.75 ± 0.13	3 ± 0.2	11.75 ± 0.06	25.25 ± 0.05	21 ± 0.07	17.5 ± 0.08	18.5 ± 0.09	20.5 ± 0.09	22.5 ± 1.7
30	1.5 ± 0.2	3.5 ± 0.11	12.75 ± 0.07	29.25 ± 0.05	29.3 ± 0.04	23.5 ± 0.06	25 ± 0.06	25.5 ± 0.06	28.5 ± 1.6
45	1.5 ± 0.13	4 ± 0.13	13.25 ± 0.08	30.25 ± 0.05	31.3 ± 0.04	23.8 ± 0.05	27.5 ± 0.08	28.5 ± 0.08	31.5 ± 1.8
60	2 ± 0.15	4.25 ± 0.14	13.75 ± 0.09	31.25 ± 0.05	32.3 ± 0.05	24.5 ± 0.05	28 ± 0.08	29 ± 0.07	32 ± 2.0
75	2 ± 0.15	4.25 ± 0.19	14.25 ± 0.08	32.25 ± 0.04	32.8 ± 0.06	25 ± 0.06	28.5 ± 0.08	29.5 ± 0.08	32.5 ± 2.1

Table S2. Intercellular EtBr accumulation in *E. amylovora* 1189 with human GIP. Relative fluorescence intensities at excitation and emission filters of 528/2 and 590/2 nm, respectively, up to 75 minutes. Average of four independent replicates ± relative standard deviation**.**

Time (min)	**PBS**	**EtBr Background**	***E. a* 1189 EtBr**	***E. a* ΔacrB-ΔtolC EtBr**	***E. a* 1189 CCCP 12.5µg/mL**	***E. a* 1189 GIP 12.5µg/mL**	***E. a* 1189 GIP 25µg/mL**	***E. a* 1189 GIP 50µg/mL**	***E. a* 1189 GIP 100µg/mL**
0	0.8 ± 0.25	2.3 ± 0.26	11 ± 0.07	11.3 ± 0.06	10.3 ± 0.06	12 ± 0.06	11.3 ± 0.07	11.3 ± 0.06	11.3 ± 0.04
15	1 ± 0.1	3.3 ± 0.18	14.3 ± 0.06	25.3 ± 0.05	27.3 ± 0.05	19 ± 0.07	20 ± 0.06	21 ± 0.08	21 ± 0.08
30	1.5 ± 0.2	3.8 ± 0.13	15.8 ± 0.06	31.5 ± 0.05	37.8 ± 0.03	29.3 ± 0.05	31.3 ± 0.05	31.3 ± 0.05	32.3 ± 0.05
45	1.5 ± 0.2	4 ± 0.13	16.3 ± 0.07	32.8 ± 0.05	38.3 ± 0.03	30.3 ± 0.06	32.3 ± 0.05	33.3 ± 0.06	34.3 ± 0.05
60	1.8 ± 0.17	4 ± 0.15	16.8 ± 0.07	34.8 ± 0.04	39.3 ± 0.04	31 ± 0.05	33.3 ± 0.07	34.3 ± 0.05	35.3 ± 0.07
75	3.3 ± 0.12	4.3 ± 0.16	17.3 ± 0.07	35.3 ± 0.04	40.8 ± 0.04	31.3 ± 0.06	34.5 ± 0.05	35.5 ± 0.05	36.3 ± 0.05

Table S3. MIC determination of human GIP to some bacterial strains.

	MIC µg/ml[a]		
Bacterial strains	**GIP**	**Chloramphenicol**	**H_2O**
E. coli TG1	>1000	0.62	>1000
E. coli Δ*acrB* Δ*tolC*	>1000	0.062	>1000
E. amylovora 1189	>1000	0.62	>1000
E. amylovora Δ*acrB* Δ*tolC*	>1000	0.062	>1000
E. coli ATCC25922	>250	-	-

[a] MIC determination in MHB medium by two-fold dilution, the assay was repeated three times in each case thereby confirming consistencies of MIC values. Differences in MIC values were only considered significant if they were at least four-fold. Chloramphenicol was used as positive control as known substrate for AcrAB-TolC efflux pump. Water used as negative control as solvent for GIP. MHB was used as a blank and MHB inoculated with test strains was used as growth control. The results of human GIP and water were similar to MHB inoculated with test strains.

Table S4. Checkerboard assay of GIP with ampicillin and FICI determination against *E. coli* ATCC 25922, n.a.: no activity.The data were analysed in 3 biological replicates.

	Without GIP	With GIP
Ampicillin	6.25 µg/mL	6.25 µg/mL
Without Ampicillin	n.a.	>250 µg/mL
FICI	-	2

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