Electronic Supplementary Information (ESI) of the paper:

Synthetic cells produce a quorum sensing chemical signal perceived by *Pseudomonas aeruginosa*

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1. Experimental Procedures

1.1 Bacterial strains, media and recombinant DNA techniques

The bacterial strains used in this study are listed in Table S1. Unless otherwise stated, all *E. coli* and *P. aeruginosa* strains were routinely grown at 37°C in Luria-Bertani broth (LB) with aeration, and antibiotics were added at the following concentration: *E. coli*, 100 µg/mL ampicillin (Ap), 10 µg/mL tetracyclin (Tc); *P. aeruginosa*, 200 µg/mL tetracyclin (Tc), 200 µg/mL kanamycin (Km).

Plasmids used or generated in this study, details on their construction and oligonucleotides used in the work are reported in Table S2 and Table S3, respectively. Preparation of plasmid DNA, purification of DNA fragments, restrictions, ligations and transformations in *E. coli* competent cells were performed with standard procedures [1]. The DNA amplification was performed by Polymerase Chain Reaction (PCR) using the GoTaq® Polymerase (Promega). All plasmids generated in this study were checked by restriction analysis and by sequencing *via* the Genechron Sequence Service (Genechron).

Table S1. Bacterial strains used in this study.

Strain	Relevant characteristic	Reference/Source
E. coli BL21 (DE3)	Strain expressing T7 RNA polymerase	Novagen
P. aeruginosa PAO1	Wild type strain	American Type Colture Collection
P. aeruginosa PAO1 Δ*rhlI*	Strain carrying a mutation in the *rhlI* gene.	University of Washington Genome Centre
P. aeruginosa PAO1 Δ*rhlI* pKD-*rhlA*	Strain carrying a mutation in the *rhlI* gene and containing the pKD-*rhlA* plasmid, in which the expression of the *luxCDABE* operon is under the control of the P*rhlA* promoter; Km^R. Biosensor that emits light in response to C4-HSL, named RepC4-*lux*.	[2]
P. aeruginosa PAO1 Δ*rhlI* P*rhlA*::mCherry	Strain carrying a mutation in the *rhlI* gene and containing the P*rhlA*::mCherry transcriptional fusion integrated into the chromosome; Tc^R. Biosensor that emits fluorescence in response to C4-HSL, named RepC4*red*.	This study

Table S2. Plasmids used in this study.

Plasmid	Relevant characteristics and plasmid construction	Reference/Source
pWM-*egfp*	Plasmid containing the *egfp* gene under the control of the T7 promoter; Ap^R.	[3]
pWM-*rhlI*	The *rhlI* gene was PCR amplified from *P. aeruginosa* PAO1 chromosome using primers FW*rhlI* and RV*rhlI*, and cloned in the pWM-*egfp* plasmid in place of the *egfp* gene by NdeI/BamHI restriction; Ap^R.	This study
pWM-EV	The *egfp* gene was removed from the pWM-*egfp* plasmid by NdeI/BamHI restriction, and the backbone plasmid was ligated after Klenow-mediated fill-in of the protruding ends; Ap^R.	This study
pKD-*rhlA*	pMS402-reived plasmid containing a transcriptional fusion between the RhlR/C4-HSL activated promoter P*rhlA* and the *luxCDABE* operon for light emission; Km^R.	[4]
MINI-CTX-1	Suicide cloning vector allowing single copy integration of genetic cassettes in a neutral site of *P. aeruginosa* chromosome; Tc^R.	[5]

MINI-CTX-P*rhlA*::*mCherry*	The *mCherry* gene was PCR amplified from the pMM45 plasmid (kindly provided by Proff. Miguel Cámara and Paul Williams, University of Nottingham) using primers FW*mCherry* and RV*mCherry*, and cloned in the MINI-CTX-1 plasmid by HindIII/SalI restriction, thus generating the MINI-CTX-*mCherry* plasmid. Then, the P*rhlA* promoter region was PCR amplified from *P. aeruginosa* PAO1 chromosome using primers FWP*rhlA* and RVP*rhlA*, and cloned in the MINI-CTX-*mCherry* plasmid by EcoRI/HindIII restriction; Tc^R.	This study

Table S3. Oligonucleotides used in this study.

Oligonucleotides	Sequence (5' – 3') [a]	Restriction site
FW*rhlI*	TATCATATGATCGAATTGCTCTCTGAAT	NdeI
RV*rhlI*	TATGGATCCTCACACCGCCATCGACAGC	BamHI
FW*mCherry*	TATAAGCTTTGGTGAGCAAGGGCGAGG	HindIII
RV*mCherry*	TATGTCGACTCACTTGTACAGCTCGTCCATG	SalI
FWP*rhlA*	TATGAATTCGCCAGAGCGTTTCGACACC	EcoRI
RVP*rhlA*	TATAAGCTTGCCGCATTTCACACCTCCC	HindIII
FW16SRT	AGTACGGCCGCAAGGTTAAA	-
RV16SRT	CCCAACATCTCACGACACGA	-
FW*ant*ART	GGCTATCACGTCAGCACAGT	-
RV*ant*ART	TTGGGCATCTCGCTGAAGAG	-
FW*PA2069*RT	TACTTCTACGGGCGCATCAC	-
RV*PA2069*RT	GATCGCTGTAGCCGTCGTAG	-
FW*lec*ART	CAGGGCAGGTAACGTCGATT	-
RV*lec*ART	CAACCCGGTATTGACCGGAA	-
FW*rhlI*RT	GACGTCTTCGCCTACCTGTG	-
RV*rhlI*RT	CCGTTGCGAACGAAATAGCG	-
FW*rhlA*RT	CATCTGCTCAACGAGACCGT	-
RV*rhlA*RT	TGCCGTTGATGAAATGCACG	-
FW*lux*CRT	TTATTGTCGCTTGGGGAGGG	-
RV*lux*CRT	GGGCAGAAAAACAAGCTCGC	-

[a] Introduced restriction sites are underlined.

1.2 Synthesis of RhlI and production of C4-HSL *in vitro* and inside synthetic cells

The transcription-translation reaction mixture was prepared as specified in Table S4. The PURE system kit has been aliquoted in small fractions and stored at -80° C. The specific components of the PURE system are provided as pre-mixed solutions labelled as "mix A" (enzymes) and "mix B" (buffer and other compounds). The reaction mixture indicated in Table S4 has been used for reactions both *in vitro* and inside synthetic cells. In the latter case, the mixture was used as I-buffer for preparing the w/o droplets, that were transformed in synthetic cells by the droplet transfer method, according to the protocol described in the paragraph 1.3. The O-solution was formed by PURE system buffer (50 mM Hepes-KOH, 100 mM potassium glutamate, 13 mM magnesium acetate, 2 mM spermidine, and 1 mM DTT, pH 7.6) and 200 mM glucose. After centrifugation, the O-solution was removed and the pellet was resuspended in 25 µL of fresh O-solution. When required, DNase or RNase enzymes

(0.4 mg/mL final concentration) were added to inhibit RhlI synthesis. All stock solutions were prepared in RNase-free water and stored at -20°C.

Table S4. Transcription-translation reaction mixture for expression of RhlI and synthesis of C_4-HSL.

Component	Volume (μL)	Final concentration
PURE system, mix A	50	
PURE system, mix B	20	
DNA template	-	8 μg/mL
DTT	-	1 mM
SAM	-	500 μM
Butyryl-CoA	-	2 mM
Sucrose	-	200 mM
RNase-free water	8	
TOTAL	100	

1.3 Preparation of liposomes-based synthetic cells

Liposomes have been prepared by the droplet transfer method [6], see Fig. S0. Since only few practical and mechanistic details on this method were described in previous papers, we optimized a previous procedure [7] in order to obtain a reproducible method for the generation of a large amount of vesicles. In detail, a 3 mM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) solution in mineral oil (code M5904, Sigma-Aldrich) is prepared by prolonged stirring (2 days). The solution can be stored at 4°C and diluted just before its use to final POPC concentration of 0.5 mM.

A defined small volume of I-solution (V_i), or I-buffer, which will be contained inside liposomes, is added to a defined volume of 0.5 mM POPC in mineral oil (V_{EM}), typically V_i/V_{EM} = 0.033. Typically, 10 μL of I-buffer are emulsified in 300 μL of POPC/mineral oil solution. The w/o emulsion is created by pipetting up and down the V_i + V_{EM} mix, slowly and regularly, until a homogeneous turbid emulsion is obtained. In a 1.5 mL test tube, a volume of POPC-containing interface oil (V_{INT}) is gently stratified over a volume of O-solution (V_o), or O-buffer, in which liposomes will be resuspended, for a defined incubation time before the preparation of the w/o emulsion. Typically, 300 μL of interfacial oil are stratified over 500 μL of O-solution. The w/o emulsion, freshly prepared, is gently poured above the interfacial volume (V_{INT}); within seconds, water droplets quickly sediment filling the whole oil volume (V_{EM} + V_{INT}). The Eppendorf tubes are immediately centrifuged at room temperature or, when needed, at 4 °C, for 10 min at 2,400*g*. After centrifugation, liposomes are generally found on the bottom of the tube. The procedure has been scaled-up in order to be carried out in Falcon tubes (V_i = 50 μL, V_{EM} = 2 mL, V_{INT} = 0.5 mL, V_O = 2 mL) and centrifuged on a swing-arm bench-top centrifuge (10 min at 2,800*g*).

The addition of sucrose and glucose in the I-solution and O-solution, respectively, facilitates the droplet transfer and the sedimentation of vesicles. That happens because the sucrose inside the droplets makes them heavy, and in this way it is easier that they cross the interface during the centrifugation. Importantly, I-buffer and O-buffer must be isotonic, in order to avoid osmotic stress. Preliminary experiments indicated that an improved protocol for liposomes preparation should be based on the use of 200 mM sucrose in the I-buffer and 200 mM glucose in the O-buffer.

Liposomes dimension and size distribution have been established by direct visualization with a TCS SP5 confocal microscope (Leica Mycrosystem, Wetzlar, Germany). Around 4,000 liposomes containing 20 μM of the fluorescent dye calcein, have been photographed and the average of the liposome radius resulted to be 3.5 μm ± 1.4 μm. The amount of liposomes produced with our optimized method has been estimated to be around 320 μM ± 60 μM, in terms of POPC concentration, determined by the Stewart assay [8]. Briefly, 100 μL of a liposomes suspension were mixed with 200 μL of ammonium ferrothiocyanate/KCl and 600 μL of chloroform. After vigorous mixing, followed by an incubation of 30 minutes, the chloroform phase was analysed spectrophotometrically by reading the absorbance at 470 nm, indicative of the POPC/ferrothiocyanate red complex. Absorbance values were converted to concentration by means of a calibration line, using POPC as standard (ε_{470} = 8.5 cm^{-1} mM^{-1}). By hypothesizing a constant radius of 3.5 μm, the number of liposomes in 1 mL of solution resulted to be around 300 million. Finally, we determined the w/o droplets transfer efficiency through the interface, as the number of droplets that successfully become liposomes. This transfer efficiency can be defined as the percentage of solutes found inside liposomes with respect to the total amount of solutes used to prepare the w/o emulsion. To calculate this percentage the water-soluble probe ferroin 2 mM (ε_{508} 11,000 cm^{-1} M^{-1}) has been used. After the formation of liposomes, the absorbance of the supernatants (A_s) was measured and compared with the maximum achievable absorbance (A_{max}), corresponding to 100 % solute release. The transfer efficiency (%), intended as the fraction of V_i found inside liposomes, is calculated as 100 × (1 – A_s/A_{max}). In our hands, the transfer efficiency of the w/o droplets resulted to be up to 75% (this value means that 75 droplets, over 100 of them, become liposomes).

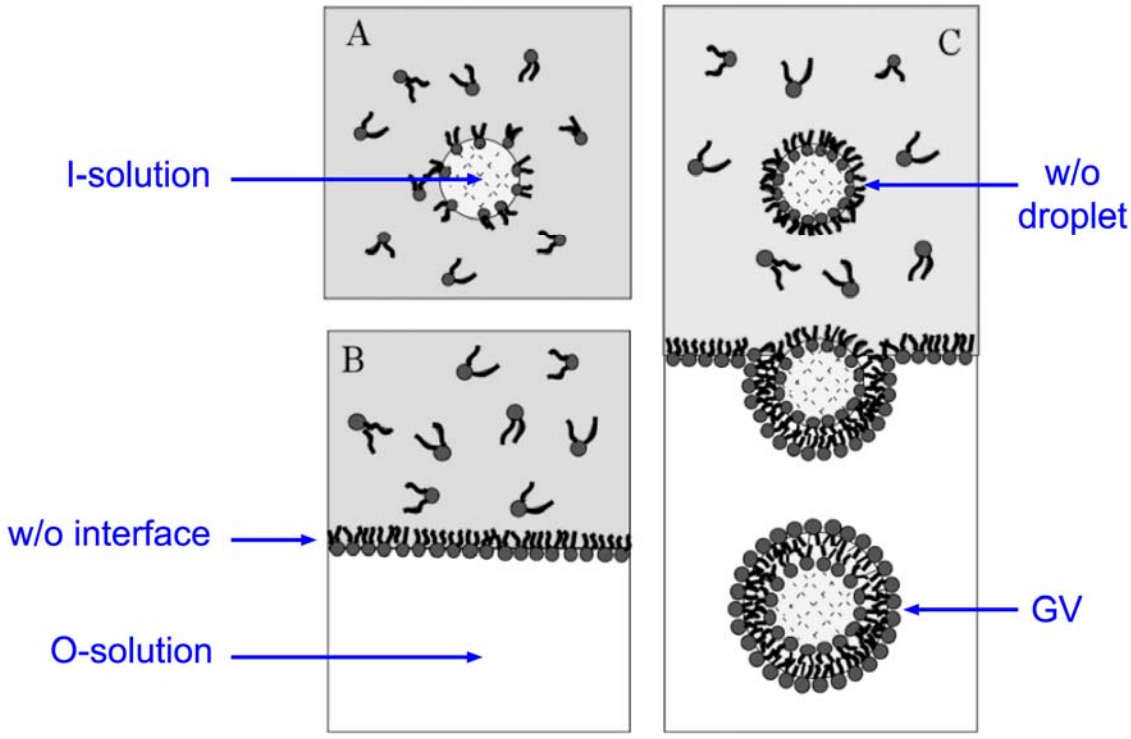

Figure S0. Preparation of GVs by the droplet transfer method (schematic). For details see textual explanation in the experimental part. Adapted with permission from [6]. Copyright 2003 American Chemical Society (in particular, the figure has been modified just by addition of the blue labels). Note that the drawn is not to scale (in particular, the shown curvature is very high; in the case of GVs, actually, the droplets curvature is negligible).

1.4 Encapsulation of C4-HSL inside GVs

C4-HSL-filled GVs were prepared by the droplet transfer method, following the procedure described in Section 1.3, with the difference that I-solution was composed by MC buffer (25 mM Tris-acetate, 100 mM potassium acetate, 1 mM DTT, pH 7.5; MC buffer is generally used in TX-TL reactions) and 200 mM sucrose, whereas the O-solution was composed by MC buffer and 200 mM glucose. Different concentrations of commercial C4-HSL was dissolved in the I-solution before emulsification. A certain amount of C4-HSL ethyl acetate solution was first placed in a vial, and the I-solution (10 μL) added after complete evaporation of ethyl acetate. The C4-HSL-filled w/o droplets were transformed into GVs by centrifugation and collected from the O-solution (500 μL). From the latter, two samples were taken for analysis: 100 μL of supernatant, and the last 100 μL that include the GVs pellet. Analysis consisted in the co-incubation, in liquid LB medium with RepC_4*lux*, as described in Section 1.5. Sodium cholate (2.5 mM) was added to similarly prepared duplicate samples.

Control experiments were run in order to measure the effect of sodium cholate on the RepC_4*lux* response (in particular, the reporter strain was co-incubated with different concentrations of C4-HSL in absence and in presence of 2.5 mM sodium cholate). Depending on incubation time and bacterial density, sodium cholate increases (+12%, 1.5 hours) or decreases (−8-13%, 3-4.5 hours) the bacterial response (data not shown).

1.5 Measurement of bioluminescence

Bioluminescence emitted from the RepC4*lux* strain was determined as a function of population density by using the automated luminometer-spectrometer VICTOR 3V (Perkin-Elmer). An LB grown over-night culture of the RepC4*lux* strain was diluted in fresh LB to an absorbance at 600 nm wavelength (A_{600}) of 2.0. One-hundred eighty μL of this culture was transferred in a microtiter plate (Perkin-Elmer), together with 20 μL of the samples to be tested, or synthetic C4-HSL as a standard. Luminescence and turbidity were determined every hour for 20 hours. Luminescence is given as Relative Light Units (RLU) normalized by cell density (A_{600}). The average data and standard deviations were calculated from three independent experiments.

1.6 Quantification of RhlI expressed inside SCs

The quantification of the expressed RhlI was performed according to the general procedure described by [9]. Briefly, [^{35}S]methionin (>37 TBq/mmol, 10 mCi/ml) was added in the reaction mixture of PURE system and allowed to icorporate into the synthesized RhlI. After the synthesis reaction, the product was subjected by SDS-PAGE and the resulting gel was dried up on a filter paper. The obtained gel image after overnight exposure was analyzed by an imager softwere, such as Image Gauge (Fujifilm). Quantitative determination was performed based on a sandard curve obtained by measuring a series of diluted [^{35}S]methionin solutions.

1.7 Gas cromatography-mass spectroscopy (GC-MS) analysis

The GC-MS analysis was performed with a Shimadzu GC2010 gas chromatograph combined with a mass spectrometer Shimadzu QP2010S. The injector was set at 250°C, the helium flow rate at 1 mL/min. A phenyl-methyl silicone capillary column it has been used, by setting the following temperature program: 150°C for 4 min,

an increase of 10°C/min until 250°C, and finally 10 min at constant 250°C. The mass spectrum was recorded through the ionization due to electrons impact at 70eV, then the mass from 40 to 200 *m*/*z* has been analyzed.

1.8 RNA extraction and Real Time PCR analysis

RNA was extracted from C4-HSL-producing synthetic cells incubated with RepC4/*lux*. Samples were treated with RNAprotect Bacteria Reagent (Qiagen), and total RNA extraction was performed with the RNeasy Mini Columns Kit (Qiagen) as the manufacturer's instructions, including the on-column DNase I digestion described by the manufacturer. In addition, RNAs were treated for 1 h at 37°C with DNase TURBO (Ambion). DNase I was removed with the RNeasy Column Purification kit (Quiagen). RNA was quantified with the NanoDrop 2000 spectrophotometer (Thermo-Fisher Scientific). cDNA synthesis was performed with the iScript Reverse Transcription Supermix for RT-qPCR kit (Bio-Rad Laboratories) according to manufacturer's instructions, and quantified with NanoDrop 2000. Real Time PCRs were performed using iTaqTM Universal SYBR® Green Supermix kit (Bio-Rad Laboratories), as the manufacturer's instructions, and Rotor Gene 6000 thermocycler (Corbett Research). Primers employed in this analysis, reported in Table S3, were designed using the Primer-blast software (www.ncbi.nlm.nih.gov/tools/primer-blast). The reaction procedures involved incubation at 95°C for 1 min and 40 cycles of amplification at 95°C for 10 sec and 60°C for 45 sec. Fluorescence was registered in the last 15 sec of the 60°C step. 16S ribosomal RNA was chosen as an internal control (housekeeping gene) to normalize the Real Time PCR data in each single run, and to calculate the relative fold change in gene expression by using the $2^{-\Delta\Delta Ct}$ method. The average data and standard deviations were calculated from three independent experiments.

1.9 Estimation of synthetic cells stability in LB and in a bacterial culture

The stability of synthetic cells in LB and in the presence of bacteria was estimated *via* the calcein dequenching assay [10]. Calcein is a membrane impermeable fluorescent dye with excitation and emission wavelengths of 495 nm and 515 nm, respectively. Above the calcein concentration of about 0.1 mM, fluorescence decreases (by self-quenching) in concentration-dependent manner. In the concentration range corresponding to the self-quenching (0.1-100 mM), calcein dilution brings about a fluorescence increase. Calcein can be entrapped inside synthetic cells at high concentration, so that its fluorescence is low. Stable synthetic cells do not release calcein, and fluorescence remains low. Unstable synthetic cells release calcein in the medium, and fluorescence increases due to the calcein dilution. Quantitative analysis of calcein release can be then correlated with synthetic cells stability. Synthetic cells containing 40 mM calcein were prepared by the droplet transfer method. After the centrifugation, the resulting pellet was washed four times with 300 µL LB, to remove non-entrapped calcein, and used immediately. Bacteria were grown over-night in LB at 37°C, washed by centrifugation (1 min at 12,000 rpm), and resuspended in fresh LB medium to a final A_{600} of 2.0. The calcein fluorescence versus time profile of synthetic cells incubated with bacteria or LB was recorded during time, at 25°C. Sodium cholate 15 mM pH 7.5 was used as detergent to solubilize the synthetic cells and release all calcein, and the corresponding fluorescence value was taken as 100% release.

1.10 Immobilization of synthetic cells and bacteria in a gel matrix

Warm, liquid-like LB-agar 30 g/L, was gently directly mixed (1:1 vol/vol) with synthetic cells/RepC4*lux* or synthetic cells/RepC4*red* mixtures. The so-obtained LB-agar gels, containing synthetic cells and bacteria, were quickly poured in a 96-well microtiter plate (Perkin-Elmer) for light emission analysis. Special microscope slides for fluorescence imaging of RepC4*red* were prepared as it follows. Freshly prepared SCs in LB-agar warm gel was deposited over a microscope slide, in the presence of flat-end cylindrical comb teeths touching the surface microscope slide. The comb teeths function as template. After gelification, the comb teeths were removed, leaving small cylindrical holes in the gel. RepC4*red* dispersed in warm LB-agar, were deposited in the holes, and allowed to gel. The so-obtained system was incubated in a humidified chamber kept at room temperature. A cover glass was applied only before the observation at the confocal microscope in order not to reduce sample aeration (which is an important parameter, as revealed by preliminary experiments).

2. Supplementary Results and Discussion

2.1 Optimization of the RepC4*lux* reporter strain

In a "synthetic-to-natural cells" communication channel, natural cells needs to fulfil specific requirements in order to be "signal negative" and able to perceive and transduce the signal molecule sent by the synthetic cells in an easily detectable and quantifiable phenotype. For this reason an appropriate reporter system has been used: a *P. aeruginosa rhlI* mutant impaired in the synthesis of the QS signal molecule C4-HSL, containing the pKD-*rhlA* plasmid [2], here called RepC4*lux* (main text, Fig. 1). The pKD-*rhlA* plasmid contains the genetic cassette P*rhlA::luxCDABE,* in which the promoter P*rhlA*, activated by the RhlR/C4-HSL complex, controls the expression of the *luxCDABE* operon. Therefore, the RepC4*lux* reporter is expected to emit light as a function of exogenous C4-HSL concentration.

In a preliminary experiment, the RepC4*lux* reporter strain was grown for 14 hours (over-night growth; O/N) in a flask at 37°C, in shacking conditions, in LB supplemented with 200 µg/mL kanamycin (Km) to positively select for plasmid maintenance. This O/N culture was diluted to reach an absorbance at 600 nm wavelength (A_{600}) of 1.0 in fresh LB medium supplemented with 200 µg/mL Km, and grown at 37°C, in shacking conditions, in the absence or in the presence of different concentrations of synthetic C4-HSL in microtiter wells. An A_{600} of 1.0 was chosen because the *rhl* QS system of *P. aeruginosa* starts to be active at this cell density [11]. Cell density and light emission from the resulting sub-cultures were collected after 4 hours of incubation. As shown in Fig. S1 (white columns) the P*rhlA* promoter activity in the RepC4*lux* strain (calculated as total light emission normalized to cell density) was proportional to the amount of synthetic C4-HSL present in the medium, proving the functionality of this reporter system for C4-HSL detection. However, the RepC4*lux* strain could not detect C4-HSL concentrations below 250 nM;. The numerical *in silico* model described in [12], predicted a production of C4-HSL by the synthetic cells of about 100-200 nM. Therefore, different experimental parameters were modified to select for experimental conditions improving the detection limit of the RepC4*lux* reporter system.

RepC4*lux* produces the *N*-3-oxododecanoyl-homoserine lactone (3OC12-HSL) QS signal, and literature data showed that this QS signal molecule may have opposite effects on the *rhl* QS system. In particular, the LasR/3OC12-HSL complex promotes the transcription of the *rhl* genes [13], while the 3OC12-HSL signal molecule decreases the transcriptional activity of the RhlR/C4-HSL complex, likely by interacting with the RhlR

receptor [11]. These literature data do not allow to discriminate between possible beneficial or detrimental effects exerted by 3OC12-HSL on RepC4*lux* sensitivity toward C4-HSL. To address this issue, we tried to add 10 µM synthetic 3OC12-HSL to the culture medium, or to decrease the concentration of this molecule by washing the over-night (O/N) culture before diluting it in the fresh medium. The exogenous provision of 3OC12-HSL did not affect the reporter response to C4-HSL (data not shown), while the washing step slightly increased its performance (Fig. S1, light grey bars). To further improve the sensitivity of the reporter system toward C4-HSL, we increased Km concentration from 200 µg/mL to 500 µg/mL, since this change could possibly select for bacteria with high-copy number of the pKD-*rhlA* plasmid, carrying the Km resistance gene. An increase in pKD-*rhlA* copy number should result in an higher intracellular concentration of the P*rhlA*::*luxCDABE* cassette, possibly decreasing the amount of synthetic C4-HSL required to ensure a measurable response of the RepC4*lux* reporter strain to this molecule. According to our hypothesis, the cultivation of RepC4*lux* at Km concentration of 500 µg/mL resulted in enhanced sensitivity of this reporter strain for C4-HSL, with respect to 200 µg/mL Km (Fig. S1, dark grey bars).

Once our experimental settings have been modified by including the washing step of the O/N culture and the addition of higher Km concentration (500 µM), we tried to further improve the reporter's performance by changing other cultural conditions, such as the medium composition (LB or M9 supplemented with glucose), the temperature (30°C or 37°C), the aeration level (static or shacking incubation), and the starting cellular density of the culture (A_{600} of 1.0, 1.5, or 2.0). This analysis revealed that the sensitivity of RepC4*lux* to C4-HSL was maximal when growing this reporter strain in LB at 30°C in static conditions, starting with an A_{600} of the culture of 2.0 (Fig. S1, black columns).

Despite each single implementation step led to slight sensitivity improvement, the overall optimization process resulted in a 8-fold decrease of the minimal concentration of C4-HSL required to activate a response in RepC4*lux*, from 250 nM to 31.25 nM (compare the white and black bars in Fig. S1). The best experimental setting here described have been applied in all the experiments described in this work, when the RepC4*lux* reporter strain has been used to quantify C4-HSL production.

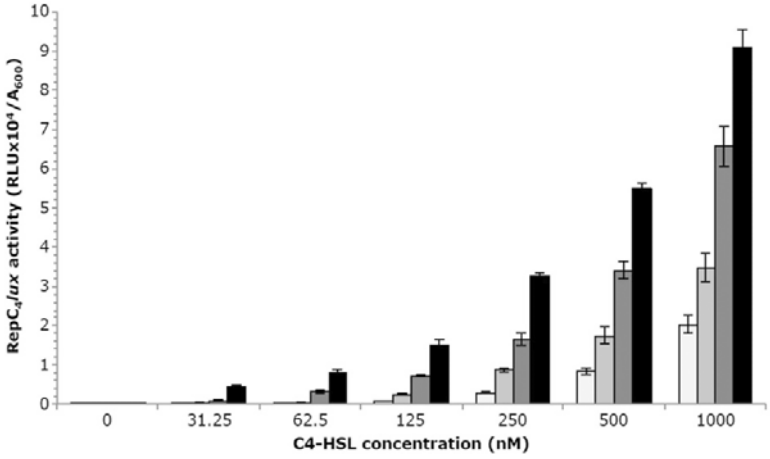

Figure S1. Graph showing the RepC_4*lux* reporter activity measured after 4 h of incubation without or with increasing concentrations of synthetic C4-HSL (indicated at the bottom). The activity is reported as relative light units (RLU) normalized to the cell density of the corresponding cultures (A_{600}). Strains were grown in LB supplemented with 200 µg/mL Km, at 37°C, in shacking conditions, starting from an A_{600} of 1.0 (white columns). The same conditions were used together with a washing step of the O/N culture (light grey bars), and with this washing step and an increased Km concentration to 500 µg/mL (dark grey bars). The maximal sensitivity of RepC_4*lux* was achieved in LB supplemented with 500 µg/mL Km, at 30°C, in shacking conditions, starting from an A_{600} of 2.0 after a washing step of the O/N culture (black bars). The average of three independent experiments is reported with standard deviations.

2.2 Construction and *E. coli*-based validation of the pWM-*rhlI* plasmid

Gene transcription *via* the PURE system is based on the T7 RNA polymerase, hence a DNA sequence encoding the *rhlI* gene under the control of a T7 RNA polymerase-dependent promoter (*i.e.* the T7 promoter) should be used as template for the PURE translation/transcription machinery. To this aim, a plasmid previously used to express enhanced Green Fluorescence Protein (eGFP) with the PURE system *in vitro* (named pWM-*egfp*) has been conveniently modified [3]. Plasmid pWM-*egfp* contains the *bla* gene for ampicillin resistance, and the *egfp* gene under the control of the T7 promoter [3]. Briefly, the the *egfp* gene contained in pWM-*egfp* was replaced by the *rhlI* gene from *P. aeruginosa* PAO1 (as described in Experimental procedures; Table S2, Fig. S2a). The resulting plasmid pWM-*rhlI* should allow the T7 RNA polymerase-dependent expression of RhlI. As a control, we also generated an empty vector, pWM-EV, by removing the *egfp* gene from the pWM-*egfp* plasmid, and by ligating the plasmid backbone upon Klenow enzyme-mediated fill-in of the restriction sites' protruding ends (as described in Experimental procedures; Table S2, Fig. S2a). To validate the functionality of the pWM-*rhlI* plasmid, both pWM-*rhlI* and pWM-EV were transformed into *E. coli* BL21(DE3) cells (Novagen), containing in the chromosome a genetic cassette for the constitutive expression of the T7 RNA polymerase. *E. coli* BL21(DE3) is not able to synthesize C4-HSL or any other QS signal produced by *P. aeruginosa*. Since the natural substrates for C4-HSL synthesis (SAM and butyryl-ACP) are common metabolites in *E. coli*, the pWM-*rhlI* plasmid is expected to confer to *E. coli* BL21(DE3) the ability to synthesize this QS signal molecule.

To verify pWM-*rhlI* functionality, the *E. coli* BL21(DE3) strains carrying the pWM-*rhlI* or pWM-EV plasmids were grow for 8 h at 37°C and the presence of C4-HSL in the corresponding cell-free spent media was evaluated by using the RepC4*lux* strain. As a positive control, a cell-free spent medium of the C4-HSL producing bacterium *P. aeruginosa* PAO1 was used. A standard curve for C4-HSL quantification was generated by simultaneously incubating the RepC4*lux* reporter strain with increasing concentrations of synthetic C4-HSL. The C4-HSL produced by the *E. coli* strain carrying the pWM-*rhlI* plasmid was about 30 µM, higher than the concentration of C4-HSL produced by *P. aeruginosa* PAO1 (about 14 µM). As expected, no C4-HSL was detectable in the spent medium of the *E. coli* strain carrying the pWM-EV plasmid (Fig. S2b). Overall, these data demonstrate that an RhlI enzyme that is active in synthesizing the QS signal molecule C4-HSL can be expressed from the T7 RNA polymerase-dependent plasmid pWM-*rhlI* in an *E. coli* BL21(DE3) genetic background.

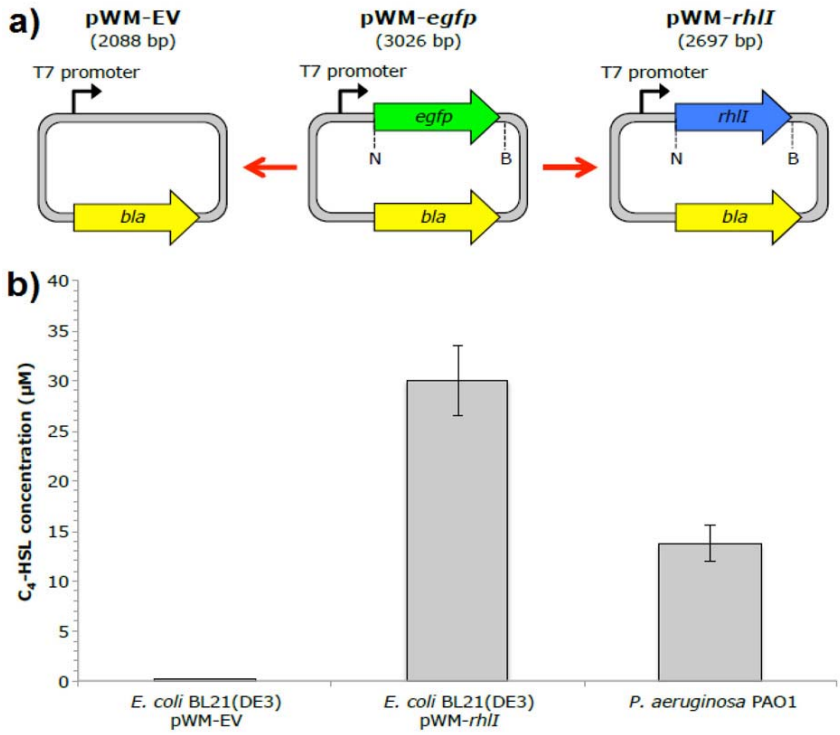

Figure S2. a) Schematic representation of the pWM-EV and pWM-*rhlI* plasmids used in this study, and of the pWM-*egfp* plasmid. *Bla*, ampicillin resistance gene; N and B, NdeI and BamHI restriction sites, respectively; bp, base pair. b) Histogram reporting the concentration of C4-HSL measured *via* the RepC4*lux* reporter strain in cell-free spent media from cultures of the indicated strains. The average of three independent experiments is reported with standard deviations.

2.3 *In vitro* synthesis of C4-HSL *via* the PURE system from a DNA template

The next step of our experimental approach was to test the possibility to synthesize C4-HSL *in vitro* from a DNA template by using the PURE system. To this aim, three independent reaction mixtures (20 µL) were prepared including the PURE system, the pWM-*rhlI* plasmid, 0.5 mM SAM and 0.5 mM butyryl-CoA (herein referred to as C4-CoA) as C4-HSL precursors. The three reaction mixtures were incubated at 37°C for 3, 5 or 7 h. In parallel, control reactions were prepared containing the pWM-EV plasmid instead of pWM-*rhlI*. After the indicated incubation times, C4-HSL concentration in the different samples was evaluated by using the RepC4*lux* reporter strain. As reported in Fig. S3, white bars, our experimental settings allowed producing ca. 1 µM C_4-HSL *in vitro* from the pWM-*rhlI* plasmid after 3 h incubation, while no C4-HSL was detectable when using the control vector pWM-EV. This shows that well-folded and therefore catalytically active RhlI can be synthesized by the PURE system. Note that the presence of the two C_4-HSL precursors (SAM and C4-CoA) does not impair the PURE system functions. Since no increase in C4-HSL production was observed for prolonged incubation times (5 and 7 h; data not shown), the following experiments were performed by incubating the C4-HSL producing SCs for 3 h.

To improve C4-HSL synthesis *in vitro* the concentration of C4-CoA was increased from 0.5 mM to 2 mM. In fact, the Michaelis-Menten constants (K_M) for the two RhlI substrates, SAM and C4-CoA, are 14 and 230 µM, respectively [14]. This means that whereas the SAM concentration (0.5 mM) is well above K_M, ensuring maximal enzyme rate, C4-CoA concentration (500 µM) was just two times the K_M. It is then expected that an increase of C4-CoA concentration brings about an increase of the reaction rate. Indeed, the use of a higher C4-CoA concentration (from 0.5 to 2 mM) led to an increase in C4-HSL synthesis to ca. 2.5 µM (Fig. S3, grey bars). This

final experiment was repeated to include a control sample with the pWM-EV plasmid. Moreover, samples containing the pWM-*rhlI* plasmid were also supplemented with DNase or RNase as negative controls, in order to degrade the pWM-*rhlI* plasmid DNA or the *rhlI* mRNA, respectively. As expected, ca. 2.5 μM C4-HSL was produced only in the sample containing the pWM-*rhlI* plasmid in the absence of DNase and RNase (Fig. S3, grey bars). According to these results, the C4-HSL biosynthetic experiments described in the following experiments were performed with 2 mM C4-CoA.

Previous studies showed that purified RhlI was able to catalyse C4-HSL synthesis from the natural precursors SAM and C4-ACP, and that synthetic C4-CoA could be used by this enzyme as an acyl donor instead of C4-ACP, even if with low efficency with respect to C4-ACP. In fact, a kinetic analysis showed that the K_M for C4-CoA was 40-fold greater than the K_M for C4-ACP [14]. Actually, the use of the unconventional precursor C4-CoA in our approach could partially account for the poor conversion of the substrates into the final product C4-HSL.

Overall, the data here described demonstrate that the synthesis of a signal molecule like C4-HSL is achievable *in vitro* with a well-defined TX-TL system of minimal complexity (*i.e.*, the PURE system) directly from adequate substrates and DNA template.

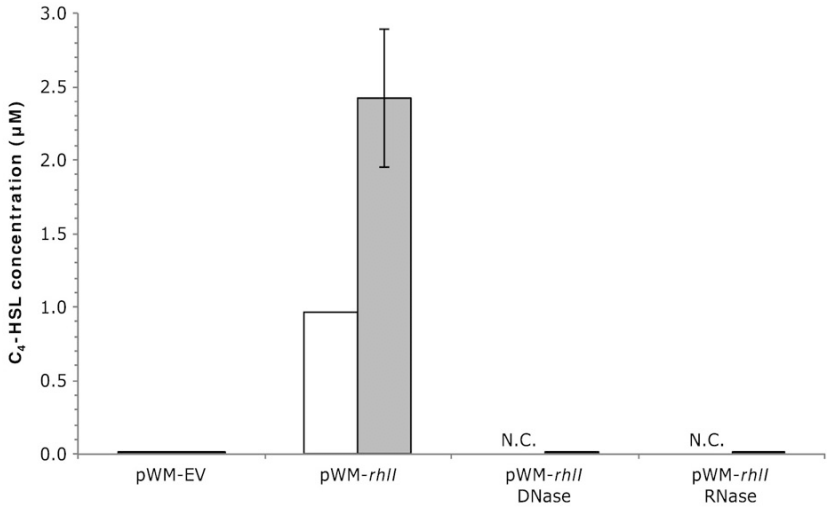

Figure S3. a) Histogram reporting the concentration of C4-HSL measured *via* the RepC4*lux* reporter strain in samples containing the PURE system, 0.5 mM SAM and the plasmid/enzyme indicated below the corresponding bar. Samples also contained 0.5 mM C4-CoA (white bars) or 2 mM C4-CoA (grey bars). N.C., data not collected. For grey bars, the average of three independent experiments is reported with standard deviations.

2.4 Characterization and validation of the communication system between synthetic and natural cells

Each step of the C4-HSL synthesis inside synthetic cells was investigated, in order to verify the transcription of *rhlI* gene into mRNA, the translation of *rhlI* mRNA into RhlI protein, *via* PURE system, and finally the identity of the signal molecule as C4-HSL.

To demonstrate the production of the *rhlI* mRNA inside SCs, total RNA from a mix containing synthetic cells and RepC4*lux* was extracted, retro-transcribed to cDNA and used in Real Time PCR analysis performed with *rhlI* gene specific primers. Please consider that *rhlI* mRNA synthesis in this system can result only from SCs, since the *rhlI* gene has been inactivated in the RepC4*lux* strain. As internal control, the housekeeping gene 16S was used. As shown in Fig. S4a, the amount of *rhlI* mRNA was about 150-fold higher in SCs containing pWM-*rhlI*

plasmid with the respect of those containing the control plasmid pWM-EV, proving the synthesis of the *rhlI* mRNA *via* the PURE system inside SCs.

To verify RhlI synthesis, protein extracts from synthetic cells containing radiolabeled ^{35}S-methionine were analyzed by means of SDS-PAGE. Autoradiography of the resulting gels revealed *de novo* synthesis of a protein with molecular weight corresponding to RhlI (main text, Figs. 2c and 2d).

Finally, to verify the identity of the signal molecule produced inside SCs, a gas chromatography-mass spectroscopy analysis was performed on ethyl acetate extracts of SCs-producing C4-HSL. The resulting *m/z* spectrum (Fig. S4b3) matches exactly with previously published spectrum of synthetic C4-HSL [15](Fig. S4b2), proving that SCs are effectively producing this signal molecule.

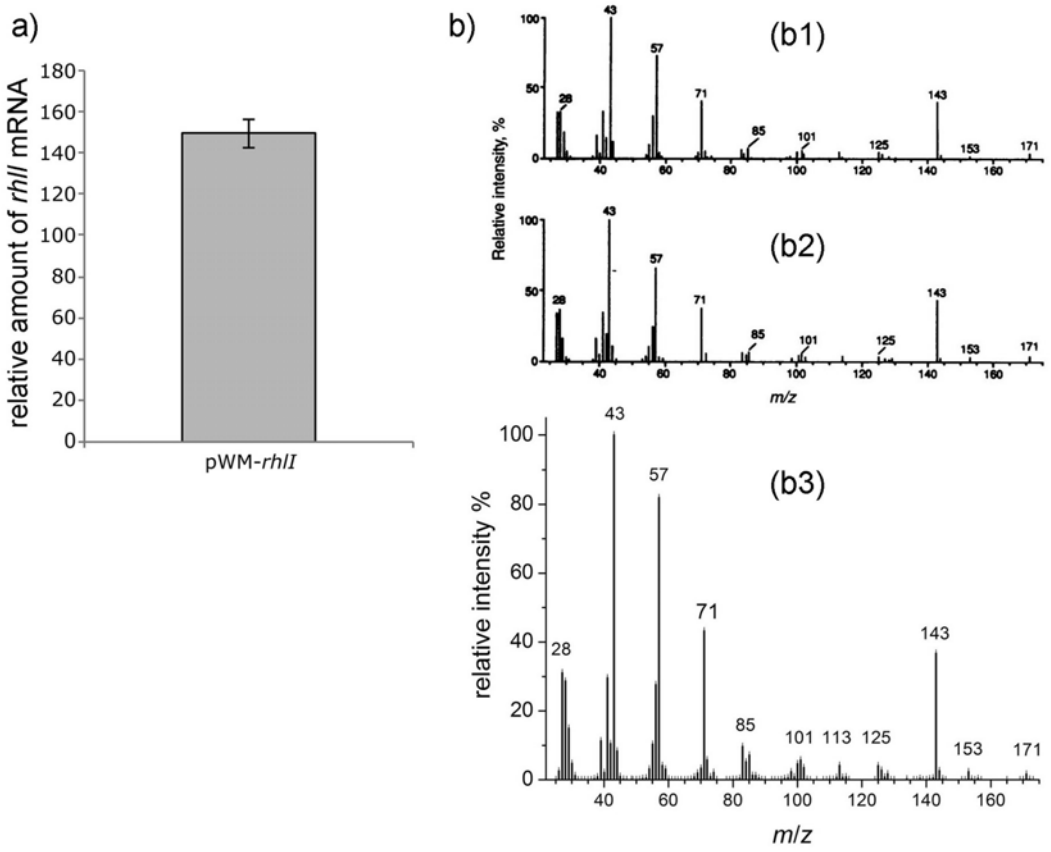

Figure S4. a) Histogram reporting the relative amount of *rhlI* RNA inside SCs containing pWM-*rhlI* plasmid with respect to SCs containing pWM-EV. The average of five independent experiments is reported with standard deviations. b) *m/z* spectrum obtained by GC-MS analysis. (b1) *m/z* spectrum of C4-HSL obtained by *P. aeruginosa* culture medium [15]); (b2) *m/z* spectrum of synthetic C4-HSL [15]; (b3) *m/z* spectrum of C4-HSL produced in this study. In particular, C4-HSL was extracted with ethyl acetate from SCs containing the PURE system, 0.5 mM SAM, 2 mM C4-CoA and pWM-*rhlI* plasmid. Panels (b1) and (b2) have been reproduced from [15] (Copyright 1995 National Academy of Sciences).

2.5 Release of C4-HSL from C4-HSL-filled GVs

The droplet transfer method allows achieving high entrapment yields. Typically, GVs contain between 50 and 70% of solutes in the sum of their inner volumes. We therefore prepared two samples (100 μL each) from the O-solution supernatant and pellet. According to scenario “a” of Fig. S5 corresponding to a low/moderate diffusion

rate of C4-HSL through the lipid membrane, the supernatant (S) should contain the "released" fraction of C4-HSL (*i.e.*, released during the droplet transfer), whereas the resuspended pellet (P) should contain a larger amount of C4-HSL mainly derived from the GVs lumen (Fig. S5a). When co-incubated with RepC$_4$*lux*, all C4-HSL becomes available, due to GVs lysis, and therefore its overall concentration could be determined as previously shown. In contrary, according to scenario "b" (Fig. S5b), corresponding to a high diffusion rate of C4-HSL through the lipid membrane, S and P should contain a very similar amount of C4-HSL, and correspondingly, produce similar biological response.

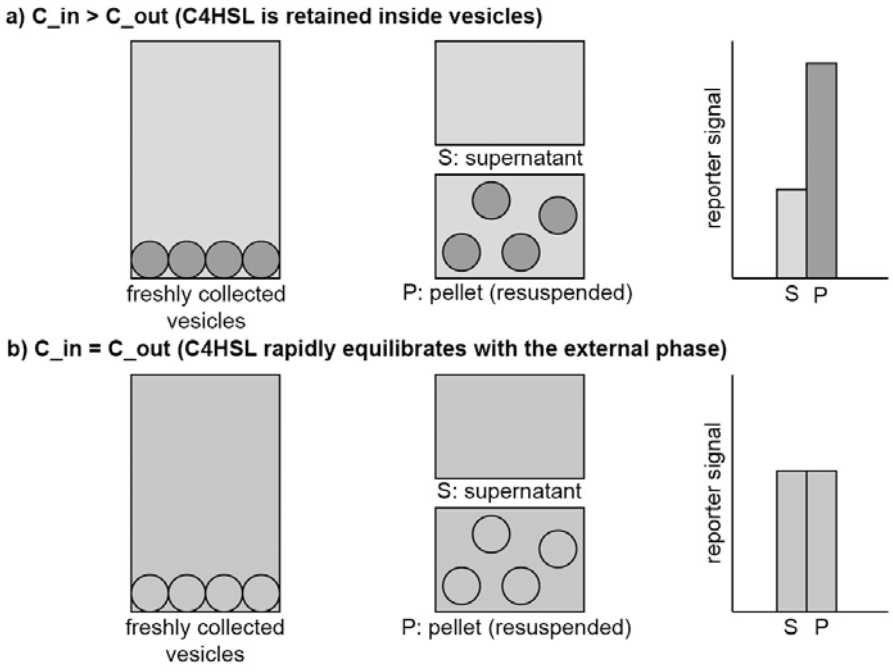

Figure S5. Experimental strategy to show the extent of C4-HSL encapsulation inside GVs. "C_in" means the intra-vesicle concentration of a certain solute, "C_out" means the external concentration. Grey colours of different darkness have been used to represent concentrations. Experimental results are shown in Fig. S6, confirming hypothesis "b".

Experimental results are shown in Fig. S6, for different incubation times and different C4-HSL concentrations. Note that S and P are run in duplicate, and sodium cholate (2.5 mM) has been added to the duplicate in order to release all C4-HSL from GVs or other small vesicles present in the supernatant, thus allowing bacteria to be exposed from the beginning to the total amount of C4-HSL in the samples. Note that sodium cholate has a minor effect on the RepC4lux response (depending on incubation time, bacterial density, and bioluminescence), but, as detailed in the experimental methods, this lies within –13 and +12%. The data support the scenario "b" of Fig. S5, in full agreement with the facile diffusion of C4-HSL through the membrane (equilibration time around 30 seconds [16]). Note that the preparation of GVs takes 10-15 minutes.

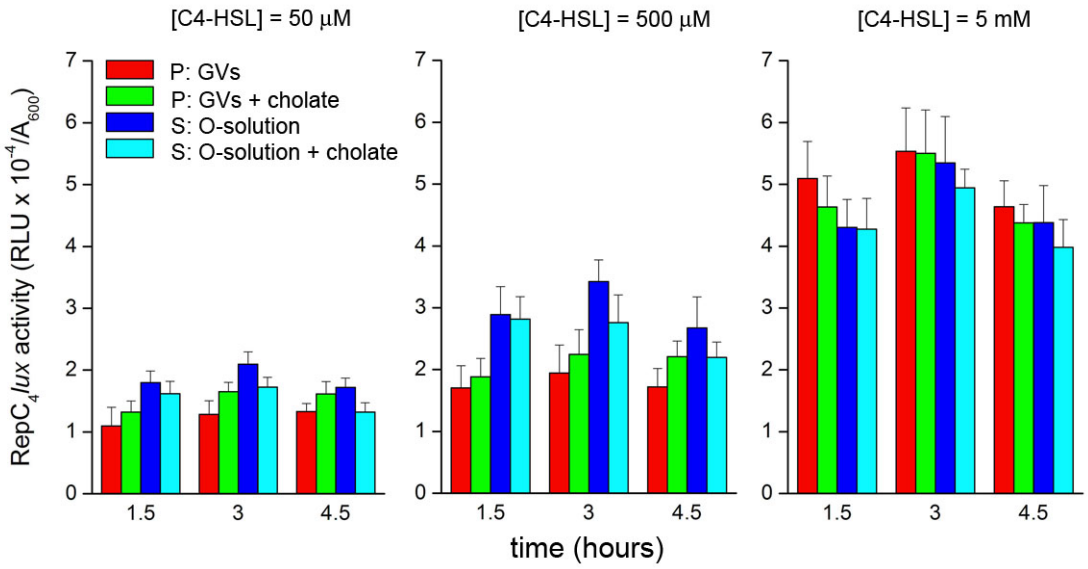

Figure S6. Experimental data referring to three different experiments, each run in duplicate (*n* = 2), on C4-HSL filled GVs prepared by the droplet transfer method. The pelletted GVs (P) and the supernatant (S), as derived from the droplet transfer method, have been co-incubated with RepC_4*lux* in order to determine the amount of C4-HSL present in each sample. Moreover, each sample was treated with 2.5 mM sodium cholate to release all C4-HSL. Please note that sodium cholate has a minor effect on the bacterial response (depending on incubation time, bacterial density, bioluminescence values) but this lies within a –13% to +12% range. In no case an enrichment of C4-HSL can be observed in the pelletted GVs, suggesting that C4-HSL rapidly equilibrates across the lipid membrane.

2.6 SCs stability in LB and in a bacterial culture (*P. aeruginosa, E. coli*)

The synthetic-to-natural communication system presented above consisted of two sequential steps: *i)* C4-HSL was produced by SCs in the absence of bacteria; *ii) P. aeruginosa* reporter cells were incubated with SCs. Ideally, the same result could be obtained in a one-step process, in which C4-HSL synthesized by SCs directly activates a response in neighbouring natural cells. This requires the co-incubation of SCs and *P. aeruginosa,* hence SCs should be stable in a bacterial growth medium and in the presence of *P. aeruginosa* cells. Therefore, we assessed SCs functionality and stability in the standard bacterial growth medium LB. To this aim, SCs containing the PURE system and the pWM-*egfp* plasmid, for the T7 RNA polymerase-dependent expression of the eGFP protein, were generated by the droplet transfer method, and the SCs pellet was immediately suspended in LB. Confocal microscope imaging of the sample revealed that SCs are stable in LB at 37°C for at least 3 h. Moreover, the eGFP protein was clearly expressed, ensuring that functional proteins can be produced inside SCs suspended in this bacterial growth medium (Fig. S7a). A spectral analysis of the emitted fluorescence confirmed eGFP identity (Fig. S7b).

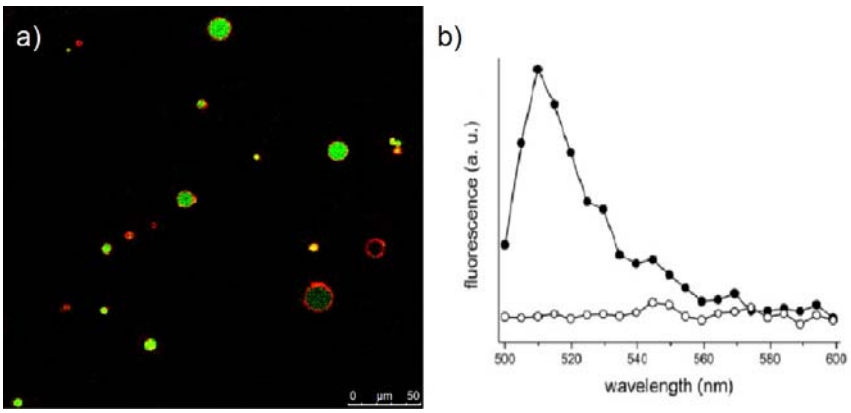

Figure S7. a) Confocal microscope image of synthetic cells expressing eGFP (green signal) in LB. The synthetic cells membrane was stained with Nile Red (red signal). b) Fluorescence emission spectrum of the green synthetic cells shown in a) as recorded by microspectrofluorimetry (expected eGFP λmax: 512 nm).

Synthetic cells stability was also assessed in more quantitative way by means of classic calcein test [10] using calcein-loaded synthetic cells as a model system. At high concentration inside SCs, calcein is self-quenched and emits low fluorescence signal; if it is released in the medium, for example as a consequence of SCs lysis, calcein is diluted, and its fluorescence emission increases (Fig. S8a). On these bases, SCs containing calcein were generated, and incubated for 3 h in LB or in a bacterial culture of the RepC4*lux* strain grown in LB. Fluorescence of the samples was measured at different time points, and sodium cholate was added to control samples to induce synthetic cells lysis; fluorescence emission from the sodium cholate containing samples was considered as 100% lysis, while basal fluorescence measured at the beginning of the experiment was considered as 0% lysis. As shown in Fig. S8b, low-fluorescence emission was measured from samples containing calcein-loaded SCs incubated in LB (maximal lysis ca. 8%), indicating that SCs are stable in LB, confirming the initial confocal microscopy qualitative observations. Conversely, the fluorescence signal increased when the calcein-loaded SCs were incubated with the RepC4*lux* culture, indicating that *P. aeruginosa* induces SCs lysis. In particular, ca. 50% lysis was induced in the first 2 h, while fluorescence emission was stable in the following hour of incubation. SCs instability induced by bacteria could be due to the crowding of *P. aeruginosa* cells in proximity of SCs (Fig. S8c).

However, the non-lysed SCs reached a steady level of ca. 50% in the bacterial culture (Fig. S8b), and such an amount of SCs could be sufficient to produce enough C4-HSL to induce a response in the bacteria. Moreover, the number of lysed synthetic cells steadily increases during the first 2 h of incubation (Fig. S8b), therefore more synthetic cells could be active in synthesizing C4-HSL in the first period of co-incubation with *P. aeruginosa*. Overall, the experiments reported above provide the indications that SCs are stable and functional in a bacterial growth medium, such as the widely used LB, while they are not entirely stable in the presence of a *P. aeruginosa* culture. However, as an alternative strategy it has been reasoned that the bacteria/SCs co-incubation could be realisable by immobilizing SCs and bacteria in a gel matrix, instead of a liquid medium. This should limit their direct contact, possibly decreasing the destabilization of SCs induced by bacteria. Moreover, the use of a low-diffusion gel matrix should lead to the local accumulation of the signal molecule nearby producer SCs, and natural receiver cells trapped in their close proximity should be exposed to high signal molecule concentration.

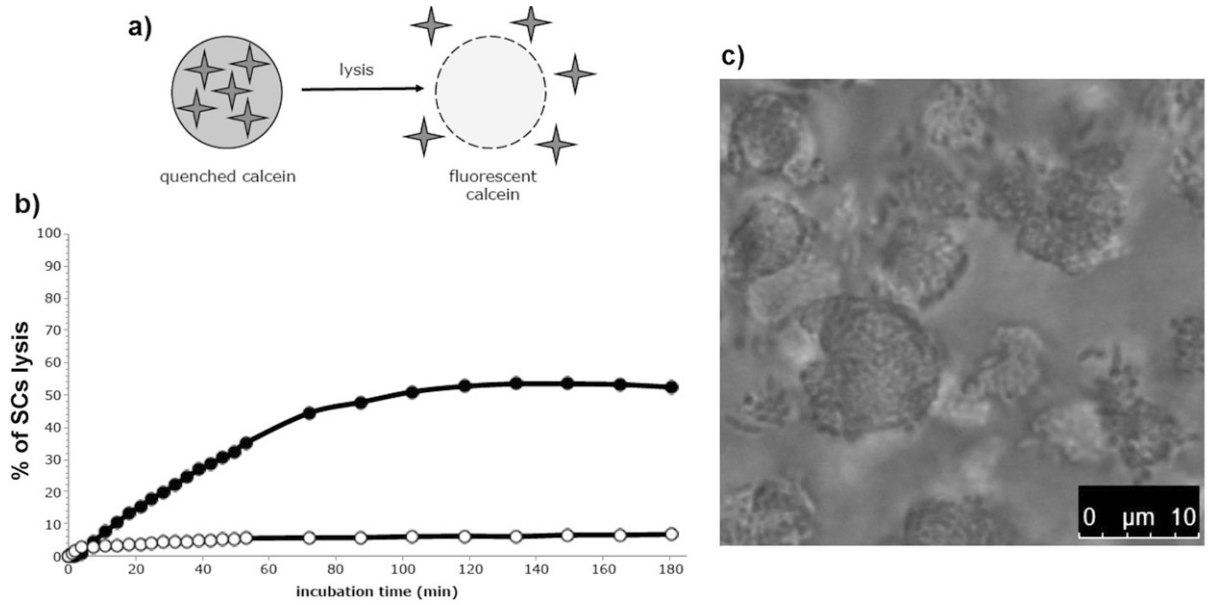

Figure S8. a) Schematic representation of the 'calcein dequenching test' used to assess SCs stability. b) Graph reporting the percentage of SCs lysis, as a function of calcein release, when incubated in LB (open circles) or in an LB-grown RepC4*lux* culture (filled circles). Maximal fluorescent emission measured upon sodium cholate addition is considered as 100% lysis. c) Bright field image, obtained by confocal microscope, of SCs containing the PURE system, co-incubated with the RepC4*lux* biosensor. Bacteria form aggregates that cover the synthetic cells.

We have also checked the stability of GVs in the presence of other bacteria, *e.g. E. coli*. As shown in Figure S9, GVs are stable, as already reported by Lentini *et al.*, 2017 [17]. Notably, the latter study also mention that *V. fischeri* and *V. harveyi*, in addition to *E. coli*, do not degrade vesicles. Therefore, we reasoned that *P. aeruginosa* behave differently due to its specific biological nature, and we ask what would be the factor determining its behavior when co-incubated with GVs (Figure S8c).

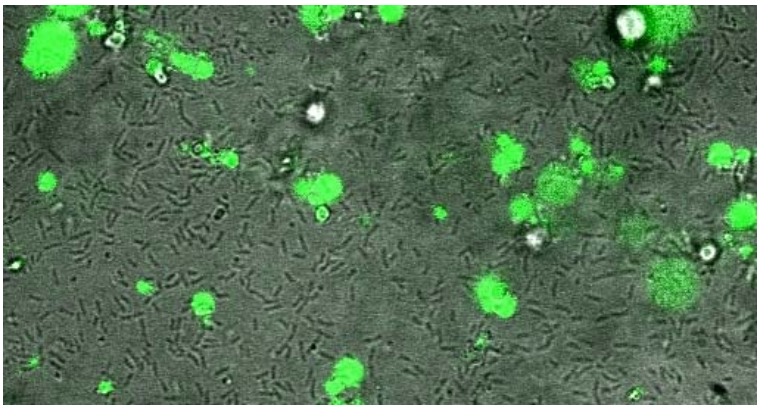

Figure S9. Calcein-containing giant liposomes (green circular particles) coincubated with *E. coli* (black rod-like particles). No vesicle/bacteria physical interaction.

A detailed investigation on the molecular basis of this interaction, although interesting, lies out of the scope of this work. However, we provide here a discussion based on available knowledge.

Rhamnolipids (Figure S10) are the best-characterized bacterial surfactants. They are produced and secreted by *P. aeruginosa* in order to make hydrophobic substances bioavailable for bacterial metabolism. These

biosurfactants are capable of emulsifying/solubilizing lipids [18, 19], justifying the disruption of GVs. Moreover, their haemolytic activity can lead to solute release from GVs (without GVs disruption). Bioavailable lipids released from GVs as a consequence of rhamnolipids activity may also act as attractants for the motile *P. aeruginosa* cells, causing crowding.

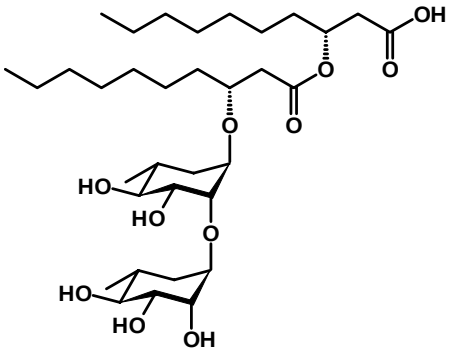

Figure S10. A typical di-rhamnolipid.

We have carried out experiments to verify whether vesicles are attacked by *P. aeruginosa* even when they do not contain the TX-TL protein-rich kit.

- We have observed that simple calcein-containing giant vesicles are also degraded by *P. aeruginosa*. This excludes a major role played by TX-TL components.

- Then we have also checked that sucrose (included inside the GVs for technical reasons) does not sustain or stimulate *P. aeruginosa* growth.

- Similarly, it can be excluded that residual oil (traces) present in GVs membrane (derived by our preparation method) is a relevant factor, because Lentini *et al.* [17] (who used an oil-free preparation method) also reported vesicles lysis by *P. aeruginosa*.

It can be concluded, in absence of further information, that crowding and lysis of GVs are specific properties of *P. aeruginosa*, likely connected to the presence of bioavailable phospholipids and involving rhamnolipids production. Indeed, it is plausible that rhamnolipids-mediated release of phospholipids from GVs activates the *P. aeruginosa* chemotactic response, causing crowding of the bacteria on GVs and their lysis.

SCs stabilization in gel

Preliminary experiments showed that SCs are stable until 4 h in a gel formed by LB growth medium in which agar is added at a concentration of 15 gr/L (LB-agar) (up to one week, if stored at 4°C, data not shown). When incubated with *P. aeruginosa* culture, recurrent is the observation in LB-agar of aggregated and/or deformed SCs. Despite this partial instability, it has been clearly demonstrated that SCs are capable of synthesizing a protein (eGFP) when co-incubated in LB-agar gel in the presence of *P. aeruginosa* (Fig. S11).

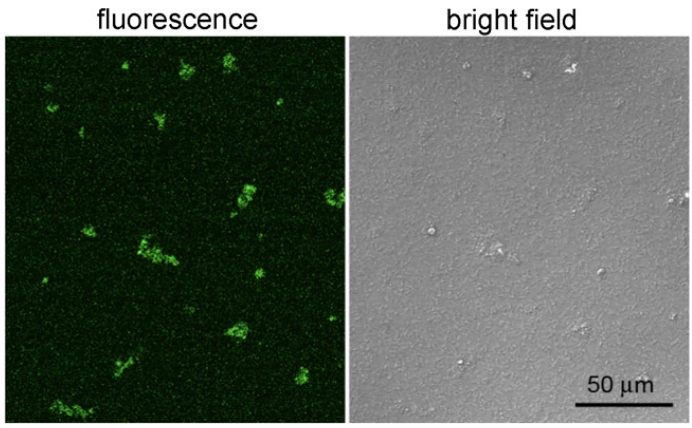

Figure S11. Image obtained by confocal microscopy of SCs capable of expressing the eGFP protein from the PURE system and the pWM-*egfp* plasmid, co-incubated with a *P. aeruginosa* culture in LB-agar.

2.7 Construction and validation of the RepC4*red* reporter strain

The immobilization of SCs and natural cells in a gel matrix described above should allow imaging of both SCs and bacteria at the single cell level, possibly increasing the detection of those bacteria activated by the signal molecule produced by the SCs. Moreover, since this approach should allow time-lapse imaging of the same natural cell, it could be useful to demonstrate the SCs to natural cells communication system “in action” at the single cell level.

To pursue this objective, a *P. aeruginosa*-based reporter strain in which C4-HSL induces the expression of the fluorescent protein mCherry was generated. Indeed, the RepC_4*lux* reporter previously described is based on light emission, a phenotype that is not possible to measure at the single cell level. Therefore, a transcriptional fusion between the P*rhlA* promoter region and the mCherry gene was generated, and it was inserted in single copy in a neutral site of the chromosome of the *P. aeruginosa rhlI* mutant strain (see Table S1 and Table S2). The resulting RepC4*red* reporter strain should emit a red fluorescence signal in response to the QS signal molecule C4-HSL. As shown in Fig. S12, this phenotype was verified by confocal microscope imaging, ensuring the functionality of the RepC4*red* reporter strain.

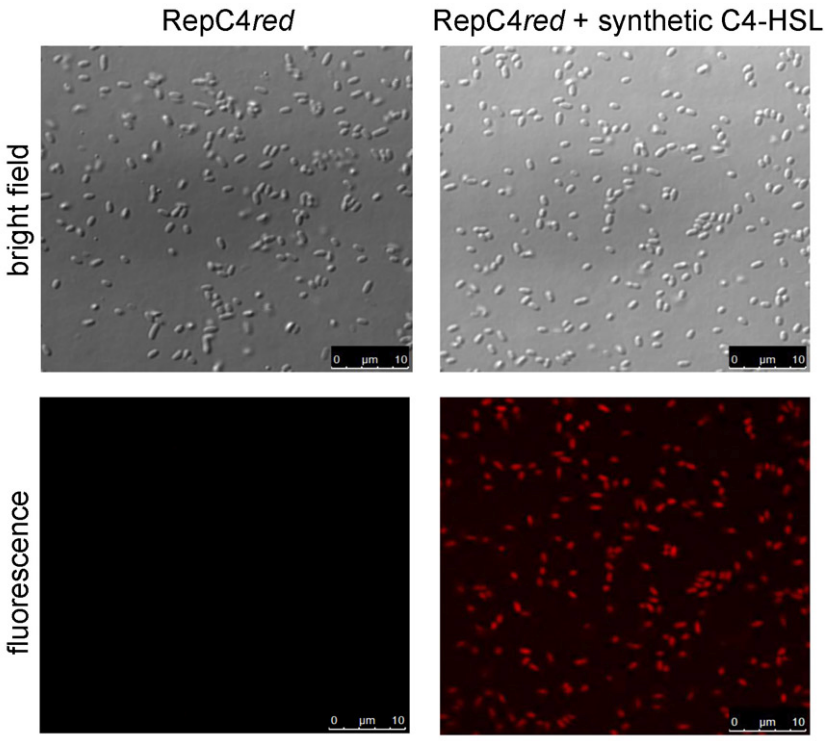

Figure S12. Confocal microscope images showing C4-HSL-induced red fluorescence emission by the RepC4*red* strain. RepC4*red* was grown in LB (left panels) or in LB supplemented with 20 µM synthetic C4-HSL (right panels).

Finally, the fluorescence properties of intra-bacterial mCherry protein (for example in experiments as those described in the main text, Fig. 3c) were verified by microspectrofluorimetry. In particular, regions hosting bacteria were submitted to λ-scan experiment in order to record the emission spectrum. As shown in Fig. S13, a remarkable agreement is observed between the emission spectra recorded in bulk (panel *a*) and in the microscope slide (panel *b*).

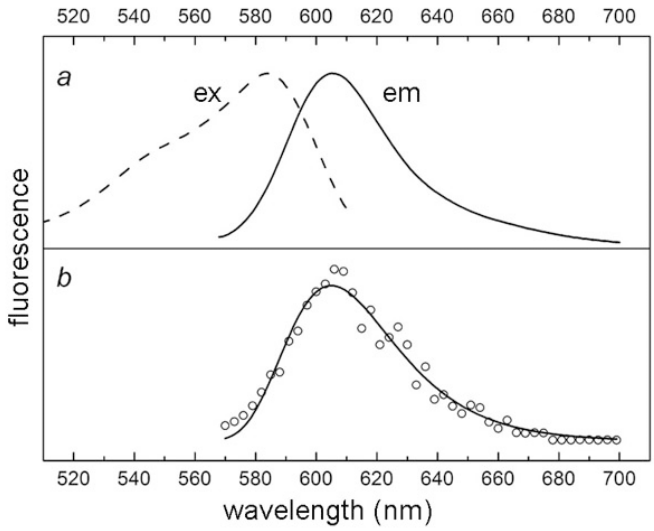

Figure S13. Fluorescence properties of mCherry. a) Excitation and emission spectra of mCherry extracted from *P. aeruginosa* treated with synthetic C4-HSL (the spectra were recorded on conventional the fluorimeter Jasco FP-6200), note that λex = 584 nm and λem = 605 nm. b) Emission spectrum of mCherry inside *P. aeruginosa* RepC4*red* in the gel matrix as reported in the main text, Fig. 3c.

2.8 Discussion on the potential development of SCs for nanomedicine

Our results, as well as those presented by other groups, elicit the question whether, and at what extent, SCs can be used as tools in nanomedicine, for example – in the context of SCs-bacteria communication, to interfere with bacterial communication, or to behave as "intelligent" drug delivery systems capable of interfacing with biological cells, feel/send signal, and behaves correspondingly. In particular, an open question is the *in vivo* stability of SCs.

Liposomes are already used in internationally approved commercial formulations for the delivery of anti-tumour and anti-fungal drugs (*e.g.*, Doxil, Ambisome), or many other formulations are in phase I, II, and III. A huge amount of studies have been devoted to understanding the fate of liposomes in vivo, in laboratory animals [20].

The factors affecting liposome stability *in vivo* (in the blood stream), are well known: (1) vesicle size (max size up to 100-150 nm), (2) lipid composition (cholesterol improves stability), (3) presence of few % PEG-ylated lipids (increase stability by reducing liposome opsonization), (4) vesicle charge (cationic lipids decrease liposome stability).

SCs are liposomes filled with macromolecules. As far as the external liposome surface does not present proteins or other biomolecules, it is expected that SCs behave as "simple" drug-containing liposomes.

Importantly, our SCs, based on GVs, cannot be directly used for *in vivo* applications, because they are too large. Future developments in this field must be based on conventional submicrometer vesicles (100-150 nm), as miniaturized versions of our SCs. This elicits more questions:

- Is it possible to build conventional submicrometer vesicles encapsulating TX-TL systems, and producing proteins? Yes, as confirmed by an already published report [21].
- Is it possible to build vesicles encapsulating TX-TL systems, whose lipids include PEGylated lipids and/or cholesterol? Yes, as confirmed by already published reports, for example [22,23].
- Is it possible to tune the lipid composition, and therefore the electrostatic charge, of vesicles encapsulating TX-TL systems? Yes, [as confirmed by already published reports, for example [24].

In conclusion, available knowledge provides motivated and well-documented reasons to state that small SCs (100-150 nm), not presenting proteins on their surface, would be stable in plasma in comparable way to current commercially available liposome formulations for drug delivery.

Our study deals with GVs only for demonstrative purposes, and because GVs can be easily studied by conventional light microscopy. GVs cannot be used for *in vivo* applications based on pulmonary or intravenous administration (but could be used, for example, for topical administration).

3. Acknowledgements and Authors Contribution

Acknowledgements. The Authors thank Pier Luigi Luisi (Univ. Roma Tre and ETH Zürich) and Luisa Damiano (Univ. of Messina) for inspiring discussions, and Miguel Cámara and Paul Williams (Univ. of Nottingham) for kindly providing the *mCherry* gene. Paolo Carrara (Univ. Roma Tre) is acknowledged for initial work on the droplet transfer method.

Author Contribution statement. P. Stano conceived the idea of chemical communication between synthetic and natural cells. G. Rampioni defined an experimental research project based on the use of *P. aeruginosa* and quorum sensing molecules such as C4-HSL. P. Stano, G. Rampioni, F. D'Angelo and L. Leoni further elaborated the experimental approach (F. D'angelo, "*Studio delle basi molecolari per lo sviluppo di sistemi di comunicazione tra batteri e cellule semi-sintetiche*", Thesis for the Bachelor Degree in Biology, University of Roma Tre, February 2012). F. D'Angelo, A. Zennaro, M. Messina, G. Rampioni and P. Stano carried out chemical communication experiments by using SCs and *P. aeruginosa*. Y. Kuruma provided the PURE system and carried out experiments on cell-free RhlI synthesis. D. Tofani identified C4-HSL by GC-MS. P. Stano, G. Rampioni, L. Leoni and F. D'Angelo wrote the paper; all authors commented and revised the paper.

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