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Outer membrane vesicles facilitate trafficking of the hydrophobic signaling molecule CAI-1 between *Vibrio harveyi* cells

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ABSTRACT

Many bacteria use extracellular signaling molecules to coordinate group behavior, a process referred to as quorum sensing (QS). However, some QS molecules are hydrophobic in character and are probably unable to diffuse across the bacterial cell envelope. How these molecules are disseminated between bacterial cells within a population is not yet fully understood. Here we show that the marine pathogen *Vibrio harveyi* packages the hydrophobic QS molecule CAI-1, a long-chain amino ketone, into outer membrane vesicles. Electron micrographs indicate that outer membrane vesicles of variable size are predominantly produced and released into the surroundings during stationary phase of *V. harveyi*, which correlates with the timing of CAI-1-dependent signaling. The large vesicles (diameter < 55 nm) can trigger a QS phenotype in CAI-1 non-producing *V. harveyi* and *V. cholerae* cells. Packaging of CAI-1 into outer membrane vesicles might stabilize the molecule in aqueous environments and facilitate its distribution over distances.

IMPORTANCE

Formation of membrane vesicles is ubiquitous among bacteria. These vesicles are involved in protein and DNA transfer and offer new approaches for vaccination. Gram-negative bacteria use among others hydrophobic signaling molecules for cell-cell communication, however due to their hydrophobic character it is unclear how these molecules are disseminated between bacterial cells. Here we show that the marine *Vibrio harveyi* packages one of its quorum sensing molecules, the long-chain ketone CAI-1, into outer membrane vesicles (OMVs). Isolated CAI-1-containing vesicles trigger a quorum sensing phenotype in CAI-1 non-producing *V. harveyi* and also in *V. cholerae* cells. Packaging of CAI-1 into OMVs not only solubilize, stabilize and concentrate this class of molecules, but facilitate their distribution between bacteria that live in aqueous environments.

INTRODUCTION

All living organisms employ various types of membrane vesicles to disseminate products into the environment. In particular, it has been known for decades that Gram-negative bacteria naturally shed 20- to 300-nm spherical vesicles from their outer membrane. Formation of outer membrane vesicles (OMVs) is ubiquitous among bacteria, and occurs in liquid and solid culture, as well as in biofilms (1, 2). Recently, extracellular vesicles (EVs) emanating from cell-walled organisms, e.g. Gram-positive bacteria, mycobacteria and fungi, have also been described (3). The surface of OMVs is thought to reflect the composition of the bacterial outer membrane, and is therefore primarily comprised of phospholipids, outer membrane proteins and lipopolysaccharides. The lumen of OMVs contains mostly periplasmic components, which are trapped within the vesicle during the release process. Surprisingly, OMVs also include proteins derived from the inner membrane, together with cytoplasmic proteins, chromosomal, plasmid and viral DNA, RNA, ions, metabolites (1, 4, 5). The biological function of these vesicles has not been fully elucidated, but it is known that OMVs are involved in protein and DNA transfer, as well as in signaling between bacteria (6). Furthermore, OMVs can also have ecological functions, as they are necessary for survival in particular environments (7). Additionally, in many pathogenic bacteria, OMVs have been shown to act as vehicles for long-distance delivery of toxins and effector proteins to host cells. For example, the main virulence factor in *Vibrio cholerae*, the cholera toxin, and colonization promoting factors are associated with OMVs, which deliver it to intestinal epithelial cells of the host (8, 9). Thereby, OMVs might be promising platforms to develop novel vaccines (10). Thus, OMVs enable the transport of toxins and effectors into host cells without any need for direct contact between pathogenic bacteria and their target host cells. Moreover, OMVs provide a means of stabilizing, concentrating and protecting bacterial molecules with specific properties until they make contact with their cognate target receptors.

Many bacterial species use low-molecular-weight molecules, such as N-acyl homoserine lactones (AHLs), for cell-cell communication in a process referred to as quorum sensing (QS) (11). In general, QS molecules are synthesized and released into the environment. They accumulate in a cell-density-dependent manner and are recognized by specific receptors, which may be located either in the inner membrane or the cytoplasm (12). Certain signaling molecules are highly hydrophobic, but the cell envelope of Gram-negative bacteria acts as an efficient barrier to the diffusion of hydrophobic molecules due to the presence of the polar lipopolysaccharide layer on the exterior of the cell (13). Hence, it is not clear how they make their way through an aqueous environment to nearby cells, and then cross the bacterial outer

membrane in order to bind to their cognate receptors in the inner membrane or the cytoplasm. Structurally diverse classes of QS molecules that regulate various phenotypes in Gram-negative bacteria have been identified over the past few decades (14). Several bacterial species use complex QS networks, with different types of signaling molecules which are sensed by specific receptors, for fine-tuning the activation of key phenotypes, such as virulence or biofilm formation (15, 16). Short-chain AHLs are thought to diffuse freely across the bacterial cell envelope, whereas long-chain AHLs are assumed to require transport mechanisms across the cell envelope, owing to their length and inherent hydrophobicity (17, 18). The length and degree of substitution of the *N*-acyl side chain therefore determine whether an AHL is freely diffusible or requires active export and import mechanisms (17).

Interestingly, up to now, few examples of signaling molecules associated with OMVs have been described. The coral-associated bacterium *V. shilonii* produces OMVs that contain alkaline phosphatase, lipase and chitinase, as well as AHLs (19). Moreover, the long-chain AHL (C16-*N*-(hexadecanoyl)-L-homoserine lactone), used for cell-cell communication in the soil bacterium *Paracoccus denitrificans* Pd1222, is mainly released via membrane vesicles (20). Furthermore, the opportunistic human pathogen *Pseudomonas aeruginosa* packages most of the Pseudomonas quinolone signal (PQS) into OMVs, and a type VI secretion effector recruits PQS-containing OMVs for iron acquisition within the population (4, 21). However, how the hydrophobic QS molecules of the CAI-1 type – typically long-chain ketones – cross the bacterial envelope (22) and reach other cells in an aqueous environment is not yet understood.

CAI-1, the predominant QS molecule of the human pathogenic bacterium *V. cholerae*, is produced by all *Vibrio* spp, albeit with different acyl chain lengths and modifications (14). Besides CAI-1 [(*Z*)-3-aminoundec-2-en-4-one, = Ea-C_8-CAI-1], *V. harveyi* strain BAA-1116 [recently reclassified as *V. campbellii* (23)] synthesizes two other signaling molecules – HAI-1, the *N*-3-(hydroxybutyryl)-homoserine lactone, and AI-2, a furanosyl borate diester (22, 24, 25). The use of AI-2 is widespread, and it is thought to be an inter-species signaling molecule (26). In contrast, HAI-1 is specific for *V. harveyi* and its close relatives (27). In *V. harveyi*, peak production of these three AIs occurs in different growth phases (28). While AI-2 is synthesized during early exponential growth, HAI-1 and CAI-1 are undetectable prior to the late exponential growth phase. Furthermore, each QS molecule is perceived by a specific membrane-integrated hybrid sensor kinase in *V. harveyi*: HAI-1 is sensed by LuxN, AI-2 by LuxQ in combination with the periplasmic binding-protein LuxP, and CAI-1 by CqsS (15, 25, 29, 30). These hybrid sensor kinases channel the information into a shared regulatory pathway.

Briefly, at low cell densities and correspondingly low signaling molecule concentrations, the sensors act as kinases and maintain the QS phenotypes in an OFF state. At high cell densities, upon perception of their cognate signaling molecule, the kinase activities are inhibited, and genes whose protein products mediate various phenotypes – such as luminescence, biofilm formation or proteolysis - are induced (31-33). Additionally, genes for type III secretion and siderophore production are repressed (15, 34).

While detailed knowledge of signal synthesis and integration in the QS cascade of HAI-1, AI-2 and CAI-1 in *V. harveyi* is now available, most studies have disregarded the potential impact of the physicochemical properties of these signaling molecules on their dissemination in aqueous media. The partition coefficient logP defines the distribution of a molecule between a lipophilic and an aqueous phase and, based on this criterion, HAI-1 and AI-2 are both hydrophilic (with LogP values of -0.94 and -1.25, respectively; for comparison, methanol has a LogP of -0.8). CAI-1 has a high lipophilicity (LogP of 3.05, which is comparable to that of dichlorobenzene) so that it readily partitions into a lipid bilayer, but once in the bilayer, it might not partition out again, and thus be unable to cross the polar lipopolysaccharide layer on the outside of the cell (13, 35). Since CAI-1 is known to function as a signaling molecule that influences the phenotypes of remote single cells within the population, it must somehow be conveyed through the aqueous environment. To obtain deeper insight into the trafficking of CAI-1, we investigated the association of CAI-1 with OMVs. Our data indicate that, when grown in complex, nutrient-rich medium, *V. harveyi* produces OMVs in the late stationary phase. Moreover, the signaling molecule CAI-1 is packaged into OMVs that are recognized by non-producing *V. harveyi* cells and activate their QS cascade.

148 **RESULTS**

149 ***V. harveyi* naturally releases OMVs in the late stationary growth phase.** *V. harveyi*
150 cultivated in the complex LM medium naturally produces OMVs, which are visible on the
151 cell surface and in the surrounding medium in stationary phase (Fig. 1A). Furthermore, TEM
152 images of ultrathin sections reveal that OMVs of *V. harveyi* are indeed derived from the outer
153 membrane of the cells (Fig. 1B, C). The OMVs produced by *V. harveyi* vary in size from
154 20 nm to 260 nm. The majority of OMVs that are shed are small, and only 7.6 % of them
155 were found to be larger than 55 nm (Fig. 1 D,E).
156
157 **CAI-1 is found in *V. harveyi* OMVs.** In order to study the impact of OMV release on
158 activation of the QS cascade in *V. harveyi*, OMVs were harvested in the late stationary growth
159 phase from *V. harveyi* MR17 strain that synthesizes only CAI-1. Cells were pelleted by
160 centrifugation (cell pellet) and the supernatant (SN) was subsequently treated with gentamicin
161 for 3 h at 30 °C to inhibit growth of the remaining cells. Afterwards the supernatant was
162 filtered through a 0.45-µm PVDF filter to obtain a cell-free fraction, which contains OMVs
163 (0.45 µm filtrate). Part of the OMV fraction was then passed through a 0.22-µm PVDF filter,
164 yielding a 0.22 µm filtrate fraction, which contains mainly small OMVs (Fig. 1E). All
165 fractions (OMVs 0.45 µm and 0.22 µm filtrate) were tested for remaining bacteria by
166 inoculating LM medium, but growth was never observed after 24 hours at 30°C. The fractions
167 were then incubated with the gentamicin-resistant *V. harveyi* reporter strains NL20 (*cqsS*$^{+}$,
168 Δ*luxN*, Δ*luxPQ*), which lack all three QS synthases (are AI^{-}) and is only able to sense and
169 respond to CAI-1, and luminescence was assed as the QS readout.
170 None of the fractions affected the growth of the reporter strain (Fig. S1). Supernatant and
171 OMV-containing 0.45 µm filtrate obtained from *V. harveyi* MR17 strain that synthesizes only
172 CAI-1 led to QS activation and therefore luminescence production in the reporter strain
173 NL20, which expresses the CqsS receptor (Fig. 2B). When this OMV-containing suspension
174 was then passed through a 0.22 µm PVDF filter, the resulting filtrate hardly contained CAI-1
175 to induce luminescence production (Fig. 2B). Similarly, OMVs isolated from a CAI-1-non-
176 producing strain (MR16, *cqsA*$^{-}$) showed no activation of the QS cascade in the reporter strain
177 NL20.
178 The *V. harveyi* MR17 cell pellet also contained only a small amount of CAI-1 as indicated by
179 15-fold lower luminescence production in comparison to the OMV-fraction of the 0.45 µm
180 filtrate (Fig. 2B). It is important to note, that large OMVs (diameter of >55 nm) were only

observed in the 0.45 µm filtrate (Fig. 1D). Those were hardly found in the 0.22 µm filtrate, but were retained on the 0.22 µm PVDF filter (Fig. 1E and Fig. S2).

Serial dilutions of OMVs suspension (0.45 µm filtrate) of MR17 show a linear increase of the reporter strain activity at low concentrations. At higher concentrations of the OMV-containing 0.45 µm filtrate (e.g. 15-21%), no further increase in QS activation was found, demonstrating the saturation of the reporter strain (Fig. S3).

CAI-1 containing OMVs activate QS in non-producing *V. harveyi* at single-cell level. We previously demonstrated that the QS cascade of *V. harveyi* is inherently noisy, which is reflected in a heterogeneous output that becomes homogenous when the available sensors are saturated with their cognate AIs (36). To study QS activation via OMVs at single-cell level, we therefore used the reporter strain NL20, which contains a chromosomally integrated fusion between the luciferase promoter (P_{luxC}) and *mCherry* at the *att*Tn*7* site (36). Fluorescence of this reporter was induced after exposure of cells to either CAI-1-containing supernatant or OMVs from strain MR17 (*csqA*$^{+}$). Furthermore, the *luxC* promoter was activated homogeneously by the OMV-containing 0.45 µm filtrate of MR17 and its supernatant (Fig. 3). These results indicate that OMVs contain sufficient amounts of CAI-1 to generate a homogenous response in this reporter strain.

In addition, we fluorescently-stained the membrane of the CAI-1-containing OMV fraction (0.45 µm filtrate) of MR17 and after thorough washing, we added these stained OMVs to the reporter strain *V. harveyi* NL20 P_{luxC}-mCherry, which reports CAI-1 dependent activation of the QS signaling cascade at single-cell level. By using microscopy, we first saw the attachment of the green-fluorescent OMVs (1 hour) and subsequent green fluorescence of the membranes of the unlabeled cells (within 2 hours). Eventually, the reporter was activated and the cells exhibited mCherry fluorescence, and thus activation of the QS cascade (2 - 4 hours) (Fig. 4).

Medium composition and growth phase affect the delivery of CAI-1 via OMVs. As previous studies have shown that CAI-1 activity peaks in the stationary phase when cells are grown in the complex LM medium (15, 28), we tested the influence of different media on the delivery of CAI-1 into OMV. OMVs were harvested at different time points from *V. harveyi* MR17 grown either in the complex and rich LM medium or in defined, minimal AB medium. Maximal activation of CAI-1-dependent QS, as measured by the high luminescence of the reporter strain NL20, was observed with OMVs harvested after 24 h when strain MR17 was

cultured in LM medium (Fig. 5A). OMVs harvested at earlier or later time points or after growth in AB medium were far less active (Fig. 5A). According to these results, more CAI-1 is produced by cells grown in the rich medium LM than in AB medium, and its level peaks at 24 h. To visualize and quantitatively determine the OMVs produced by *V. harveyi* MR17, we stained them with a fluorescent lipid-specific dye. Staining of the lipid of OMVs provides a better measure of OMV yield, as the phospholipid content is less influenced by the growth phase than the protein content (37). OMV abundance itself was found to be high in stationary phase, i.e. at 24 h and 48 h, and higher overall after cultivation in LM than in AB medium (Fig. 5B). These results reveal that CAI-1 production coincides with OMV release. In parallel, we did not find a stimulation of OMV production after external addition of CAI-1 (Fig. S4).

Inter-species communication. Previously, it was demonstrated that CAI-1 (Ea-C_8-CAI-1) produced by *V. harveyi* is recognized as a QS signal by *V. cholerae* (15). We therefore tested the ability of OMV-associated CAI-1 from *V. harveyi* to activate QS in a *V. cholerae* reporter strain. Although external addition of the *V. cholerae* (*Vc*)-specific CAI-1 [(*S*)-3-hydroxytridecan-4-one] or its endogenous synthesis induced markedly higher luminescence production in the *V. cholerae* reporter, OMVs isolated from *V. harveyi* MR17 grown in LM (5-fold lower) were indeed able to detectably activate QS-dependent luminescence reporter activity in *V. cholerae* (Fig. 6).

DISCUSSION

Production of membrane vesicles is a ubiquitous process among bacterial species, and occurs in biofilms, liquid and solid culture (1, 2). Here we have demonstrated that *V. harveyi* naturally releases outer membrane vesicles (OMVs) into its environment during stationary phase (Fig. 1). Furthermore, CAI-1 was found to be associated with OMVs leading to activation of the QS cascade in a CAI-1-non-producing strain (Fig. 2B). These OMVs can activate the QS cascade homogeneously in all CAI-1-non-producing cells (Fig. 3). Thus, *V. harveyi* OMVs can be used as a vehicle for trafficking of CAI-1 between cells (Fig. 3 and 4). Moreover, the amount of CAI-1 in OMVs was sufficient to saturate the corresponding QS-receptor (Fig. S3), which results in a homogeneous QS-response in *V. harveyi* cells (36).

Growth conditions undoubtedly have an influence on the numbers, size, content and lipid as well as protein composition of OMVs produced. Thereby, the released OMVs can contain different properties influencing differently neighboring cells (2). Likewise, the amount of CAI-1 associated with *V. harveyi* OMVs was found to be dependent on growth phase and medium composition (Fig. 5). *V. harveyi* released OMVs predominantly in rich medium and at stationary phase (Fig. 5B). Moreover, the CAI-1 content of *V. harveyi* OMVs varied as well, and peaked at early stationary phase in rich medium (Fig. 5A). The *Pseudomonas* quinolone signal (PQS) is required for membrane vesicle formation and seems to dictate OMV biogenesis (38, 39). By contrast, exogenous CAI-1 itself does not stimulate OMV formation in V. harveyi (Fig. S4).

Previously, OMV production was shown to be influenced by antibiotic treatment, preferentially by envelope targeting or DNA damaging antibiotics (40). For examples, in *P. aeruginosa* OMV formation is increased upon treatment with ciprofloxacin, an antibiotic which leads to DNA damage and results therefore in the activation of the SOS response (41). Since in our case filtration (0.22 µm filter) removed not only all cells but also the large CAI-1 containing OMVs, we used gentamicin to kill the remaining cells in the 0.45 µm filtrate (Fig. 2A). Although gentamicin inhibits protein synthesis by binding to the bacterial 30*S* ribosomal subunit (42), it cannot be completely ruled out that this treatment might cause additional release of OMVs. However, the number of remaining cells in the 0.45 µm filtrate was very low.

The CAI-1-specific receptor CqsS is located in the inner membrane, and the incoming lipophilic CAI-1 molecule probably interacts with intramembrane segments of the receptor dimer, as extracellular loops for ligand binding are absent (43, 44). Conversely, due to its inherent lipophilic character, CAI-1 is unlikely to diffuse across the polar lipopolysaccharide

layer (13) on the outside of the sending cell. It probably remains trapped in the outer and/or inner membrane, which would potentially make it available for incorporation into OMVs. In this form, CAI-1 can be easily transported between cells and can activate the QS cascade in neighboring cells (Fig. 7). In rich medium, external CAI-1 is first detected in stationary phase (28), which coincides with the onset of OMV formation. OMVs produced by *V. harveyi* range from 20 nm to 260 nm in diameter (Fig. 1), similar in size to those released by many other bacteria (2). The majority of OMVs that are shed by *V. harveyi* are small (diameter < 50 nm), however about 10 % of them are large (diameter 55-260 nm), and only those were able to activate CAI-1 dependent QS. It is still unclear, whether only the large OMVs are able to fuse with the surface of neighboring cells.

CAI-1 is a *Vibrio*-specific, interspecies signaling molecule used by several *Vibrio* species, although it mediates opposing effects on their QS cascades. Interestingly, *V. harveyi* CAI-1 can control gene expression in *V. cholerae* and *vice versa* (15). Indeed, OMVs released by *V. harveyi* MR17, which synthesizes only CAI-1, is able to activate QS-dependent luminescence reporter activity in *V. cholerae* (Fig. 6). Recently, it was shown that OMVs produced by *P. denitrificans* Pd1222 fuse with varying affinities to different bacterial species (20).

Overall, delivery of CAI-1 via OMVs might provide three major advantages: (i) solubilization and stabilization of CAI-1 in an aqueous environment, (ii) increasing concentration of CAI-1, and (iii) the unhindered passage of CAI-1 across the polar lipopolysaccharide layer of both the producing and targeted cell.

291 **MATERIALS AND METHODS**

292 **Bacterial strains.** The *V. harveyi* strains MR17 (Δ*luxM* Δ*luxS*), MR13 (Δ*cqsA*), MR16
293 (Δ*cqsA* Δ*luxM*) and NL20 (Δ*luxM* Δ*luxS* Δ*cqsA* Δ*luxN* Δ*luxQ* P_{luxC}-mCherry) (36) were
294 cultivated in Luria marine (LM) medium (20 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract) or
295 autoinducer bioassay (AB) medium (45) and incubated aerobically on a rotary shaker at 30°C
296 (36). Media used for the strain NL20 were supplemented with 20 µg/ml gentamicin.
297 The plasmid pBAD-*cqsA* used to enable synthesis of CAI-1, and the control plasmid pCMW-
298 1 (46), were introduced into the *V. cholerae* strain BH-1575 (Δ*cqsA* Δ*luxS*) by conjugation.
299 The resulting strains were additionally conjugated with the pBB1 plasmid, which carries the *V.*
300 *harveyi luxCDABE* operon (47), in order to use luminescence production as a read-out for QS.
301 These *V. cholerae* reporter strains, named KPS754 (BH-1575 pBAD-*cqsA* pBB1) and
302 KPS755 (BH-1575-pCMW-1 pBB1), were grown at 37 °C in LB (5 g/l NaCl, 10 g/l tryptone,
303 5 g/l yeast extract) supplemented with 50 µg/ml kanamycin and 5 µg/ml tetracycline. For
304 construction of pBAD-*cqsA* (pKP-420), *cqsA* was amplified from *V. cholerae* genomic DNA
305 by PCR using primers KPO-0776P (5'-gtcagctggcgttaaattttt-3') and KPO-0777 (5'-
306 gtttttggtacccttaggaataacgtttagcag-3'), and cloned into the KpnI site in pKP-331 (48). Correct
307 insertion was verified by sequence analysis.
308
309 **Preparation of OMVs**. For OMV preparation, an overnight culture of *V. harveyi* MR17,
310 MR16 or MR13 was adjusted to an OD_{600} of 0.05 in LM or AB medium and incubated at
311 30 °C for 6, 24 or 48 h with shaking at 250 rpm (adapted from (49)). The *V. harveyi* strain
312 MR17 produces only CAI-1, whereas the strain MR13 or MR16 do not produce CAI-1. To
313 isolate OMVs from each of these strains, after growing the cells they were first pelleted at
314 4 °C by centrifugation at 5,000 rpm for 1 h. The supernatant was then treated with 60 µg/ml
315 gentamicin at 30 °C for 3 h to inhibit the growth of any remaining cells, which are
316 gentamicin-sensitive (in contrast to the gentamicin-resistant reporter strain). This supernatant
317 was then filtered through a 0.45-µm filter (PVDF Membrane, Merck Millipore) to obtain a
318 cell-free suspension that contains OMVs (0.45 µm filtrate). This OMV-containing suspension
319 was additionally fractionated via filtration through a 0.22-µm filter (PVDF Membrane, Merck
320 Millipore). Equal volumes were maintained for all collected fractions during the preparation
321 of OMVs (see Fig. 2A for an overview).
322 OMV-containing suspensions prepared for the *V. cholerae* reporter were treated similarly,
323 except that 150 µg/ml kanamycin and 15 µg/ml tetracycline were added instead of gentamicin.
324 In order to follow the impact of CAI-1 on OMV formation, *V. harveyi* strain MR13, which

does not synthesize CAI-1, was cultivated in LM at 30°C for 24 h with shaking at 250 rpm and then incubated for 1 and 2 h with externally supplied (10 µM) *V. cholerae*-specific *Vc* CAI-1 [(*S*)-3-hydroxytridecan-4-one] (44). OMVs were then harvested as described above.

Labelling of OMVs. Isolated OMVs were stained with the MitoTracker Green FM dye (ThermoFisher), which is non-fluorescent in aqueous solution but becomes fluorescent in the presence of phospholipids. Staining with this marker provides a better measure of OMV yield, as the phospholipid content is less influenced by the growth phase (37). A stock solution (1 mM) of the dye was prepared in DMSO and OMVs were incubated with 0.5 µM MitoTracker Green FM for 40 min at 30 °C and washed with phosphate-buffered saline (PBS, pH 7.4). After centrifugation at 35,000 rpm at 4°C for 1 h, the OMV-containing pellet was resuspended in the same volume in 1 x PBS and spotted on 1 % (wt/vol) agarose pads before microscopy. Images were taken on a Leica DMi8 microscope with a Leica DFC365 FX camera. An excitation wavelength of 485 nm and a 510 nm emission filter with a 75-nm bandwidth was used for GFP fluorescence. In order to exclude the presence of adventitiously fluorescent particles, OMV samples were additionally analyzed for red fluorescence using an excitation wavelength of 546 nm and a 605-nm emission filter with a 75-nm bandwidth (data not shown).

Bioluminescence assay and fluorescence microscopy. In order to monitor the impact of OMVs on the delivery of CAI-1 produced by *V. harveyi*, the reporter strain NL20 was used, which expresses the cognate receptor for CAI-1 and therefore can recognize only CAI-1. NL20 additionally caries a chromosomally integrated fusion between the luciferase promoter (P_{luxC}) and *mCherry* (at the *att*Tn*7* site), which allows one to monitor QS activation at the single-cell level in terms of luminescence production [RLU] as the QS ON phenotype (36). The *V. harveyi* reporter strain NL20 was grown to an OD_{600} of 0.5 in LM medium and then OMVs and the other fractions were added to a final concentration of 15%. Bioluminescence and growth were determined every 15 min in microtiter plates with a Tecan Infinite F500 system (Tecan) for 0.1 s. In order to quantitatively analyze QS activation of the reporter strain NL20, different volumes of the OMVs-0.45µm filtrate were added (0-21%).

The *V. cholerae* reporter strains KPS755 and KPS754 were cultivated in LB at 37°C and bioluminescence and growth were determined every 15 min in microtiter plates with a Tecan Spark10M system (Tecan) for 0.1 s. *V. cholerae*-specific, *Vc* CAI-1, (*S*)-3-hydroxy-tridecan-4-one (44), was dissolved in 100% DMSO and used as the positive control. The *V. cholerae*

359 reporter strains KPS755 and KPS754 were conjugated with the pBB1 plasmid, which carries
360 the *V. harveyi luxCDABE* operon, so that sensing of CAI-1 could be quantified using
361 luminescence as read-out. Data are reported as relative light units (RLU) in counts per second
362 per milliliter per OD_{600}.
363 For phase-contrast and fluorescence microscopy, samples were analyzed on 1 % (wt/vol)
364 agarose pads, which were placed on microscope slides and covered with a coverslip. Images
365 were taken as described above. An excitation wavelength of 546 nm and a 605-nm emission
366 filter with a 75-nm bandwidth was used for mCherry fluorescence. The *V. harveyi* reporter
367 strain NL20 was grown to an OD_{600} of 0.5 in LM medium and then OMVs and the other
368 fractions were added to a final dilution of 1:15. Fluorescence images of the *V. harveyi*
369 reporter strain NL20 were taken after 2, 4.5 and 6 h of incubation with the respective OMV
370 fractions. Fluorescence intensities of single cells were quantified using ImageJ (50) and are
371 displayed in arbitrary units (AU = mean fluorescence/area).
372 In order to observe attachment of OMVs to *V. harveyi* cells, the OMV-containing 0.45 µm-
373 filtrate fraction was stained with the MitoTracker Green FM as described above. The reporter
374 strain NL20 was subsequently incubated with this OMV fraction with a final dilution of 1:15
375 and incubated for up to 4 h at 30 °C. Every hour samples were taken and analyzed on 1 %
376 (wt/vol) agarose pads, which were placed on microscope slides and covered with a coverslip.
377 Images were taken as described above. An excitation wavelength of 546 nm and a 605-nm
378 emission filter with a 75-nm bandwidth was used for mCherry fluorescence in order to follow
379 the behavior of the reporter strain NL20. An excitation wavelength of 485 nm and a 510 nm
380 emission filter with a 75-nm bandwidth was used for GFP fluorescence in order to visualize
381 fluorescently-stained OMVs.
382
383 **Electron microscopy.** *V. harveyi* cells were cultivated in LM for 24 h. Cells and OMVs were
384 harvested as described above and subsequently fixed with 2.5% (vol/vol) glutardialdehyde in
385 50 mM sodium cacodylate, 2 mM $MgCl_2$, pH 7.0, at room temperature for 1 h; then rinsed
386 several times in fixative buffer and post-fixed with 1% (w/v) osmium tetroxide in fixative
387 buffer at room temperature for 1 h. After two washing steps in distilled water, the cells were
388 stained *en bloc* with 1% (w/v) uranyl acetate in 20% (vol/vol) acetone for 30 min.
389 Dehydration was performed with a graded acetone series. Samples were then infiltrated and
390 embedded in Spurr's low-viscosity resin. Ultrathin sections were cut with a diamond knife
391 and mounted on uncoated copper grids and stained with lead citrate. Micrographs were taken
392 with an EM 912 transmission electron microscope (Zeiss, Oberkochen, Germany) equipped

with an integrated OMEGA energy filter operated in the zero-loss mode.

For scanning electron microscopy drops of the sample were placed on glass slides, covered with a coverslip and rapidly frozen with liquid nitrogen. The coverslip was removed with a razor blade and the slide was immediately fixed with 2.5% (vol/vol) glutardialdehyde in 50 mM cacodylate buffer (pH 7.0) post-fixed with osmium tetroxide, dehydrated in a graded series of acetone solutions and critical-point dried from liquid CO_2, mounted on stubs, and coated with a 3-nm layer of platinum using a magnetron sputter coater. The specimens were examined with a high-resolution field-emission scanning electron microscope (Zeiss Auriga workstation) operated at 1 kV.

Diameters of isolated and attached OMVs were measured from scanning electron micrographs using ImageJ (50) of at least 1400 vesicles of different replicates. OMVs were classified into two groups with diameters < 50 nm or > 51 nm. The surface areas of OMVs were calculated by assuming them to be spherical ($s = \pi * d^2$).

Analysis of the molecular character of autoinducers (AIs). Structures of *V. harveyi* signaling molecules CAI-1, HAI-1 and AI-2 (22) were analyzed with respect to their lipophilicity (LogP) using the online tool ALOGPS 2.1 from the Virtual Computational Chemistry Laboratory (http://www.vcclab.org) (35).

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559 **Legends**

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561 **Fig. 1: *V. harveyi* naturally produces and releases OMVs.** (**A**) Scanning electron
562 micrograph of whole cell of *V. harveyi* with attached OMVs (Scale bar = 500 nm). (**B**, **C**)
563 Transmission electron micrographs of ultrathin sections of *V. harveyi* cells showing OMVs
564 formed from the outer membrane (Scale bar = 100 nm). (**D**) Scanning electron micrograph of
565 the OMV-containing 0.45 µm filtrate obtained from MR17. Isolated OMVs vary widely in
566 size (Scale bar = 200 nm). (**E**) Scanning electron micrograph of the OMV-containing 0.22 µm
567 filtrate obtained from MR17 size. (Scale bar = 200 nm). (**D**, **E**) Orange arrows indicate
568 examples of OMVs with a diameter of >55 nm.

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571 **Fig. 2: OMV-associated CAI-1 activates QS cascade in *V. harveyi* reporter strain at**
572 **population level.** (**A**) Schematic depiction of OMV harvesting process by fractionating of the
573 *V. harveyi* supernatant. (**B**) Each fraction from MR17 (Δ*luxM*, Δ*luxS*) was tested for QS
574 activation in the reporter strains NL 20 (ratio of OMVs to reporter strain culture of 1:6.7) by
575 measuring luminescence as readout. The reporter strain NL20 senses only CAI-1 and MR17
576 synthesizes only CAI-1. As control, the OMV-containing 0.45 µm filtrate of the non-CAI-1
577 producing strain MR16 (Δ*cqsA* Δ*luxM*) was tested for QS activation in the reporter strains NL
578 20. Error bars represent the standard deviations of data from three different experiments.
579 RLU, relative light units, expressed in counts per second per ml per OD_{600}. SN, culture
580 supernatant of MR17; OMVs (0.45 µm filtrate), filtrate of SN of MR17 through 0.45 µm
581 filter; OMVs (0.22 µm filtrate), filtrate of SN of MR17 additionally through 0.22 µm filter;
582 LM - cell free culture medium as control; OMVs CAI-1$^-$, filtrate of SN of MR16 through 0.45
583 µm filter. Time courses of the growth and luminescence production of the reporter strain
584 NL20 are shown in Fig. S1.

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586

587 **Fig. 3: Homogeneous OMV-mediated CAI-1 response in *V. harveyi* at the single-cell**
588 **level.** The *V. harveyi* reporter NL20 (AI$^-$, *cqsS*$^+$, Δ*luxN*, Δ*luxPQ*, P_{luxC}-mCherry) was grown
589 to an OD_{600} of 0.5, supernatant or OMVs were added as indicated (see also text), and cells
590 were analyzed for expression of the P_{luxC}-*mCherry* reporter fusion after further growth for 4.5
591 h (left panel; PH, phase contrast images; mCherry, fluorescence images; crude SN, culture
592 supernatant of MR17; OMVs (0.45 µm filtrate), filtrate of SN of MR17 through 0.45 µm

filter; OMVs (0.22 µm filtrate), filtrate of SN of MR17 additionally through 0.22 µm filter; OMVs CAI-1$^-$, filtrate of SN of MR16 through 0.45 µm filter.) Average fluorescence of 1000 cells quantified by ImageJ [arbitrary unit = AU] is presented in the graphs on the right. Scale bar = 5 µm.

Fig. 4: CAI-1 is delivered via OMVs to non-producing cells and triggers intracellular QS activation. The membrane of CAI-1 containing OMVs [OMV-producing strain MR17 (Δ*luxM*, Δ*luxS*)] was fluorescently-stained, and green fluorescent OMVs were detected in the microscope (upper panel). These OMVs were incubated with the non-fluorescent reporter strain *V. harveyi* NL20 (AI$^-$, *cqsS*$^+$, Δ*luxN*, Δ*luxPQ*, P_{luxC}-mCherry). At the indicated times cells were analyzed for green fluorescence and expression of the P_{luxC}-*mCherry* reporter fusion by microscopy (lower panels). (PH, phase contrast images; green fluorescence, green fluorescence images; mCherry, red fluorescence images). Scale bar = 5 µm.

Fig. 5: Delivery of CAI-1 into OMVs depends on growth factors. (**A**) The NL20 reporter strain, which responds only to CAI-1, was incubated with OMVs harvested from *V. harveyi* MR17 (0.45 µfiltrate). OMVs were harvested after 6, 24 and 48 h of growth in LM or AB medium. QS activation in the reporter strain NL20 was monitored by measuring luminescence. Error bars represent the standard deviations of data from three different experiments. RLU, relative light units, expressed in counts per second per ml per OD_{600}. (**B**) Images of fluorescently labelled OMVs of MR17 harvested after 6, 24 and 48 h of growth in LM or AB medium. Isolated OMVs were stained with the green fluorescent MitoTrackerFM dye, which stains lipids, and imaged using a green fluorescent channel. Each white arrow indicates a single fluorescent OMV and the black arrow shows its respective position in the phase-contrast image. PH, phase-contrast images; green fluorescence images of stained OMVs. Scale bar = 5 µm.

Fig. 6: CAI-1 in OMVs from *V. harveyi* activates QS cascade in *V. cholerae*. The *V. cholerae* reporter strains KPS754 (Δ*luxS*) and KPS755 (Δ*cqsA*, Δ*luxS*) carry the pBB1 plasmid, which harbors the *V. harveyi luxCDABE* operon, enabling *V. cholerae* to produce luminescence. The *V. cholerae* reporter strain KPS755 was incubated with different

concentrations of *Vc* CAI-1 dissolved in DMSO and with different OMV-containing fractions harvested from *V. harveyi* MR17 grown in either LM or AB medium for 24 h. QS activation in these reporter strains was followed by monitoring luminescence production. Error bars represent the standard deviations of data from three different experiments. RLU, relative light units expressed in counts per second per ml per OD_{600}.

Fig. 7: Model of trafficking of CAI-1 via OMVs between *V. harveyi* cells. Due to its high lipophilicity, CAI-1 remains trapped in the outer membrane of *V. harveyi* cells and gets incorporated into OMVs. In this form, CAI-1 can be easily transported between cells and can activate the QS cascade in neighboring cells upon recognition by its specific receptor CqsS. The other QS signaling molecules of *V. harveyi*, AI-1 and HAI-1, are freely diffusible across the cell envelope and are sensed by their specific receptors LuxPQ and LuxN, respectively, located in the inner membrane. The AIs HAI-1, CAI-1 and AI-2 are depicted with circles in pink, blue or green, respectively. AI-synthases are depicted in star-like shape in the cytoplasm. The AI-specific receptors LuxN, CqsS and LuxPQ are depicted by rectangles in pink, blue or green, respectively, connected to grey pentagons. The image was created by Christoph Hohmann (Nanosystems Initiative Munich).