Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms

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Many bacteria produce extracellular and surface-associated components such as membrane vesicles (MVs), extracellular DNA and moonlighting cytosolic proteins for which the biogenesis and export pathways are not fully understood. Here we show that the explosive cell lysis of a sub-population of cells accounts for the liberation of cytosolic content in Pseudomonas aeruginosa biofilms. Super-resolution microscopy reveals that explosive cell lysis also produces shattered membrane fragments that rapidly form MVs. A prophage endolysin encoded within the R- and F-pyocin gene cluster is essential for explosive cell lysis. Endolysin-deficient mutants are defective in MV production and biofilm development, consistent with a crucial role in the biogenesis of MVs and liberation of extracellular DNA and other biofilm matrix components. Our findings reveal that explosive cell lysis, mediated through the activity of a cryptic prophage endolysin, acts as a mechanism for the production of bacterial MVs.
The rate of transition from the rod to round cell morphotype is extremely rapid occurring in <5–10 s (Fig. 1b; Supplementary Movie 1). We analysed the survival times of 150 *P. aeruginosa* PAK round cells and found that 86% of these survived for <60 s with 35% surviving for <5 s, although some round cells had much longer survival times (Fig. 1d–f; Supplementary Movie 2). The bacterial cell wall is the primary stress-bearing structure that dictates and maintains cell shape and protects the cytoplasmic membrane against turgor and lysis13,14. Our observations suggest that the transition from rod to round cell morphotype involves the rapid loss of structural integrity of the cell wall peptidoglycan, which in most instances results in immediate explosive cell lysis. We investigated the viability of round cells by culturing *P. aeruginosa* interstitial biofilms in the presence of the live-cell impermeant nucleic acid stain ethidium homodimer-2 (EthD-2) which is a sensitive marker of dead microbial cells as it only enters cells with damaged membranes13,14. We found that round cells excluded EthD-2 indicating that they have intact membranes and are likely to be viable until the explosion event (Supplementary Movie 3). Our analyses also revealed that many of the rod cells that subsequently became round cells were frequently longer than the surrounding cells, including some extremely long cells that were up to 3–4 times longer than the average size of the neighbouring rod cells (Fig. 1g). Of 156 rod cells that became round cells, 33 were clearly undergoing cell division. These observations suggest that some round cells originated from rod cells that were either undergoing cell division or were blocked in cell division.

To determine if explosive cell lysis was a conserved phenomenon in *P. aeruginosa* strains, we cultured interstitial biofilms of the common laboratory strains PAK, PAO1, PA103, PA14, ATCC27853, five CF clinical isolates and two non-CF clinical isolates, and quantified the frequency of round cells and sites of eDNA release as a marker for explosive cell lysis. To quantify the frequency of round cells in *P. aeruginosa* interstitial biofilms, randomly selected fields of view of the interstitial biofilm monolayer were imaged with phase contrast microscopy and analysed via computer vision to identify cells and categorize their morphotypes as rod or round cells. Round cells were observed in all strains, although the frequency of round cells varied from about 1 per 3,000 to 1 per 100,000 rod cells in different strains (Supplementary Fig. 1a). Similar frequencies of punctate eDNA sites were observed in the interstitial biofilms of all strains (Supplementary Fig. 1b). These observations suggest that while eDNA release through explosive cell lysis occurs in many *P. aeruginosa* strains, the survival times of round cells appear to vary between strains that is reflected in the number of round cells visible in the population at any instant in time.

Explosive cell lysis is induced by stress. Interestingly, we noted that fluorescence imaging in the presence of the eDNA stain TOTO-1 to detect eDNA release events yielded more frequent explosive cell lysis events than when fluorescence imaging was not involved (Fig. 2a) although the process appeared identical (Supplementary Movie 4). This suggests that phototoxicity due to exposure to high-intensity excitation light stimulated explosive cell lysis. To determine if other sources of exogenous stress induce explosive cell lysis, we examined the effect of exposing *P. aeruginosa* interstitial biofilms to antibiotic stress (ciprofloxacin; CPFLX) or genotoxic stress (mitomycin C; MMC). We found that as the biofilm approached CPFLX or MMC gradients, round cells were induced and large quantities of eDNA released through explosive cell lysis (Fig. 1b). To determine if induction of explosive cell lysis by these exogenous stressors is conserved, we cultured interstitial biofilms of *P. aeruginosa* strains in the presence of penicillin G (PG) or streptomycin (SM), and monitored the induction of round cells. These experiments demonstrated that explosive cell lysis was induced by both penicillin and streptomycin to a similar extent to CPFLX and MMC (Supplementary Fig. 2).
stresses is mediated by the RecA-mediated SOS stress response regulon of *P. aeruginosa*, we examined the response of a PAO1ΔrecA mutant and found no evidence of explosive cell lysis in this strain under either inducing or non-inducing conditions (Fig. 2c).

**Lys endolysin mediates explosive cell lysis.** The cell rounding and explosive cell lysis events that we have described here appear very similar to the release of lytic bacteriophages from host cells. Moreover, DNA-damaging agents such as MMC or CPFLX are known to induce prophages in a RecA-dependent manner. The *P. aeruginosa* PAO1 genome contains a cluster of genes that encode the R- and F-type pyocins that are cryptic prophages related to the lytic bacteriophages P2 and lambda, respectively. The R- and F-pyocin gene cluster encodes the only putative bacteriophage-like endolysin that we could identify on the PAO1 genome (PA0629, previously termed lys). Interestingly, Nakayama et al. have shown that overexpression of PA0629 in *P. aeruginosa* causes cell lysis and have proposed a model in which the holin Hol (PA0614) disrupts the inner membrane thereby allowing the endolysin Lys (PA0629) to translocate to the periplasm where it degrades the peptidoglycan to release the pyocins. As is common with many prophages, the production of the R- and F-pyocins is induced through the RecA-mediated SOS response of *P. aeruginosa*. We therefore explored the possibility that the putative endolysin Lys may be responsible for eDNA release through explosive cell lysis in *P. aeruginosa* biofilms. We found that PAO1Δly and PAKΔlys mutants were significantly abrogated in the explosive cell lysis-mediated release of eDNA in interstitial biofilms under both inducing and non-inducing conditions (Fig. 3a,b). Explosive cell lysis was restored with wild-type lys provided in trans but not by the mutant allele lys that encodes
an E51V substitution in the putative active site (Fig. 3b,c; Supplementary Fig. 2), indicating that the endolytic activity of Lys is critical for explosive cell lysis in these interstitial biofilms. Furthermore, PAK\textsuperscript{D}lys interstitial biofilms lacked the intricate trail networks that are a characteristic feature of wild-type PAK \textit{P. aeruginosa} interstitial biofilms (Fig. 3a) and appeared morphologically similar to PAK interstitial biofilms cultured in the presence of DNaseI 7. This indicates that Lys-mediated explosive cell lysis is likely to be the major source of eDNA that is required for self-organization of these interstitial biofilms.

To further examine the correlation between pyocin gene expression and explosive cell lysis, we utilized a \textit{Phol-eGFP} transcriptional fusion. We followed 74 explosive cell lysis events and found that all of the exploding cells had high levels of eGFP expression compared with the neighbouring rod-shaped cells indicating that the expression of pyocin genes is upregulated in these cells (Fig. 3d; Supplementary Movie 5). We also examined the involvement of genes encoding structural components of the R- and F-pyocins and found that none of the other pyocin gene mutants examined showed any defect in eDNA release in interstitial biofilms (Supplementary Fig. 3). Taken together, these observations indicate that the pyocin endolysin Lys, but not pyocins \textit{per se}, is required for eDNA production via explosive cell lysis in interstitial biofilms of \textit{P. aeruginosa}.

### Lys mediates eDNA release in submerged biofilms

We have shown previously that eDNA plays an essential role in the early stages of the development of \textit{P. aeruginosa} biofilms that are formed on abiotic surfaces submerged in liquid nutrient media\textsuperscript{9}. To determine if explosive cell lysis accounts for eDNA release during the initial stages of the development of submerged biofilms, we performed live-cell imaging of the very early stages of biofilm development and observed the formation and explosion of round cells in both the planktonic phase and at the surface (Fig. 4a; Supplementary Movie 6). In contrast, we were unable to observe any round cells or explosive cell lysis events in PAO1\textsuperscript{D}lys.

To explore the role of explosive cell lysis in mediating eDNA release during the development of submerged biofilms, we examined biofilm formation by wild-type PAO1 and the endolysin mutant PAO1\textsuperscript{D}lys after 8 h of culture. PAO1 produced numerous microcolony structures that stained with the eDNA binding dye FL +FL.

![Figure 2](image-url) **Figure 2 | Stress induces explosive cell lysis.** (a) Frequency of explosive cell lysis events in the absence (− FL) or presence (+ FL) of fluorescence imaging, mean ± s.e.m., ∗∗∗∗ P < 0.0001, Unpaired t-test with Welch’s correction. (b) Phase-contrast (top) and TOTO-1-stained eDNA (green, bottom) of \textit{P. aeruginosa} PAO1 interstitial biofilms cultured in the presence of filter discs saturated in water, MMC or CPFLX; scale bar, 5 μm. (c) Phase-contrast (left) and TOTO-1-stained eDNA (green, right) of \textit{P. aeruginosa} PAO1 and PAO1\textsuperscript{ΔrecA} interstitial biofilms cultured in the presence of filter discs saturated in water, or MMC; scale bar, 20 μm.
stains EtHD-2 (Fig. 4b,c) or TOTO-1 (Supplementary Fig. 4a). Surface-attached round cells were also visible at this time point (Fig. 4b inset). PAO1Δlys was found to be severely defective in biofilm formation and showed no microcolony structures or round cells (Fig. 4b,c). Wild-type PAO1 biofilms cultured in the presence of DNaseI, produced no microcolonies confirming a requirement for eDNA in submerged biofilm development under these assay conditions (Fig. 4b,c). The defects in microcolony development and round cell formation in PAO1Δlys could be complemented with lys provided in trans (Fig. 4b,c). The defects in micorcolony development and round cell formation in PAO1Δlys could be complemented with lys provided in trans (Fig. 5d; Supplementary Fig. 4b). These observations indicate that explosive cell lysis mediated via the endolysin Lys is responsible for the release of eDNA required for the formation of submerged biofilms by P. aeruginosa.

Interestingly, we found that the addition of exogenous P. aeruginosa PAO1 genomic DNA to the culture media was not able to effectively restore biofilm formation to PAO1Δlys and instead significantly inhibited microcolony formation in wild-type PAO1 (Fig. 4e). These observations suggest that eDNA needs to be provided in high concentrations at the substratum to initiate biofilm formation and/or there are other components released through explosive cell lysis that are also required for the development of P. aeruginosa biofilms.

Explosive cell lysis mediates MV biogenesis. Despite the importance of bacterial MVs in various processes and their ubiquitous distribution in nature, the underlying molecular mechanisms of MV biogenesis are not well understood. We noted during live-cell imaging of P. aeruginosa interstitial biofilms cultured in the presence of a fluorescent membrane stain, the presence of numerous highly dynamic fluorescent particles (Supplementary Movie 7). Fast three-dimensional-structured illumination super-resolution microscopy (3D-SIM) revealed...
that these particles were a mixture of MVs and membrane fragments many of which were linked in chains and remained tethered to neighbouring cells (Fig. 5a; Supplementary Movie 8). We measured the sizes of 268 MVs located in situ in live P. aeruginosa biofilms that could be clearly visualized by f3D-SIM as vesicular and found these ranged in the size from 110 to 800 nm with the majority having sizes of 150–300 nm in diameter (Fig. 5b). Note, as bacterial MVs have been reported to range from 50 to 250 nm (refs 1,2), it is likely that the membrane particles observed with f3D-SIM are MVs that are smaller than the resolution limit of this imaging technique (110 nm).

P. aeruginosa biofilms have been shown to contain MVs that interact with eDNA 3,22. We used f3D-SIM to examine the localization of eDNA and MVs in live P. aeruginosa interstitial biofilms. This revealed that sites of eDNA release were often situated in areas that also contained abundant MVs (Fig. 5c), which suggests that the process of MV production and explosive cell lysis may be linked temporally and spatially. As we have determined that the endolysin Lys is required for eDNA release, we used f3D-SIM to determine if PAO1Δlys also showed a deficiency in MV production in interstitial biofilms. We found that this strain produces very few MVs compared with wild-type PAO1 (Fig. 5d) indicating that Lys is required for both eDNA release through explosive cell lysis and MV biogenesis in interstitial biofilms.

In Gram-negative bacteria, MVs are thought to be produced through blebbing of the outer membrane1. It is conceivable that the transition from rod-shaped to round cells caused by the putative peptidoglycan hydrolase activity of the Lys endolysin is concomitant with weakening of the connection between the outer

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**Figure 4 | Explosive cell lysis is required for microcolony development in submerged hydrated biofilms.** (a) Time series of the initial stages of PAO1 biofilm development 1h after inoculation showing attachment of a rod cell, its transition to round cell morphotype and subsequent explosion releasing eDNA (TOTO-1, green). Time in min (top right); scale bar, 5 μm. (b,c) Microcolonies in 8-h submerged biofilms of PAO1 (upper), PAO1Δlys (middle), and PAO1 cultured in the presence of DNasel (lower). (b) Representative phase contrast (left) and eDNA (EthiD-2, right) images; scale bar, 10 μm. Inset shows magnified view of round cell at arrow-head (c) Microcolonies in 8-h submerged biofilms per mm², n = 30. Mean ± s.e.m. *P < 0.0001, unpaired t-test with Welch’s correction. (d) Microcolonies per mm² in 8-h submerged hydrated biofilms of PAO1 and PAO1Δlys carrying either pJN105 or pJN105lys, n = 30. Mean ± s.e.m. #P < 0.0001, unpaired t-test with Welch’s correction. (e) Microcolonies per mm² in 8-h submerged hydrated biofilms of PAO1 and PAO1Δlys cultured in the absence or presence of exogenous DNA (exDNA), n = 20. Mean ± s.e.m. #P < 0.0001, unpaired t-test with Welch’s correction.
biomembrane and the peptidoglycan of the cell wall and could therefore be associated with the release of MVs. To examine the biogenesis of MVs by *P. aeruginosa* we used f3D-SIM to follow MV production in live interstitial biofilms to ascertain if bleeding occurs during or after round cell transition. Surprisingly, f3D-SIM revealed that MVs did not appear to be formed through membrane bleeding from either rod-shaped or round cells, but instead were derived from shattered membrane fragments that were produced as a consequence of explosive cell lysis (Fig. 6a,b; Supplementary Movies 9 and 10). The rate of vesicularization of membrane fragments produced through cellular explosions was extremely rapid and often too dynamic to capture by f3D-SIM, which requires objects to be stationary within the period of each image acquisition (\(1 \text{s}\)). However, we were occasionally able to capture the formation of MVs that formed more slowly and found that these were formed via the curling and self-annealing of membrane fragments produced after explosive cell lysis (Fig. 6b; Supplementary Movie 10).

*P. aeruginosa* MVs have been reported to contain a variety of cellular components including DNA, peptidoglycan and proteins derived from the outer membrane, periplasm, inner membrane and cytoplasm. As both cytoplasmic proteins and DNA are efficiently released into extracellular milieu at sites of explosive cell lysis (Fig. 1b,c), we hypothesize that the packaging of MV cargo could be accounted for by a mechanism in which cellular content that had been released through cell lysis is captured by membrane fragments as they self-anneal into MVs. Indeed, f3D-SIM of live *P. aeruginosa* interstitial biofilms showed that MVs in the interstitial biofilms of a *P. aeruginosa* strain that expresses cytoplasmic mCherry fluorescent protein contain this protein (Fig. 6c).

Furthermore, we found that when *P. aeruginosa* interstitial biofilms were cultured in the presence of the eDNA stain EthHD-2 and visualized with f3D-SIM, MVs were observed to contain EthHD-2-stained DNA within the vesicle lumen (Fig. 6d). As EthHD-2 does not enter round cells and only interacts with eDNA after its release into the environment (Supplementary Movie 3), the presence of EthHD-2-stained eDNA within the lumen of MVs supports the idea that following the release of eDNA through explosive cell lysis, some eDNA fragments are captured during MV vesicularization.

Our observations have shown that explosive cell lysis mediated by the endolysin Lys is required for MV biogenesis in *P. aeruginosa* biofilms. We also examined the role of Lys in producing MVs in planktonic culture. Interestingly, PAO1Δlys showed similar levels of MV production as the wild type under normal oxic growth in liquid medium (Fig. 7a). However, the importance of Lys in MV production was evident under conditions that stimulate MV production through induction of the SOS response such as anoxic growth or exposure to MMC (Fig. 7b,c). We found that when either recA or lys was inactivated, stress-induced MV formation was also greatly impaired whereas inactivation of pyocin structural components (pyocin tail, tail fibre or tail sheath) had no effect on MV formation (Fig. 7b,c). The defects in MMC-induced MV production by PAO1Δlys could be rescued by coexpression with the wild-type lys but not the catalytic lys* mutant allele (Fig. 7d). Promoter-reporter eGFP fusions show that PrecA, PprecA and PprP were also significantly induced by exposure to MMC (Supplementary Fig. 5).

MVs from *Escherichia coli* (*E. coli*) and *Prochlorococcus* have recently been shown to contain extracellular RNA24,25. We have found that MVs from *P. aeruginosa* also contain RNA (Fig. 7e; Supplementary Fig. 6). The presence of intact 16S and 23S rRNAs in these MVs suggests that the MV-associated RNA is not rapidly degraded (Supplementary Fig. 6). We speculated that the MV-associated mRNA may indicate the physiological state of the cells at the time the MVs were produced. A comparison of the mRNA abundances in MVs and planktonic cells obtained from oxic planktonic cultures (under non-inducing conditions) revealed that MVs are highly enriched for mRNAs that are typically expressed as part of the SOS response in *P. aeruginosa* following exposure to oxidative stress, DNA-damaging agents or antibiotics such as CPFLX20,21 (Fig. 7e; Supplementary Fig. 7e,f; Supplementary Data 1). In addition to mRNA of key regulators of the SOS response, including lexA and recA, transcripts of all genes of the R- and F-pyocin region (between PA0610 and PA0648 with the exception of PA0611) were greatly increased relative to planktonic cells. We also sequenced the DNA present in purified MVs and found that the sequences obtained covered the entire genome (Supplementary Fig. 6g), consistent with our hypothesis that eDNA fragments are captured by vesicularizing membranes.

The finding that planktonic MVs are enriched for certain mRNAs, including SOS-stress response and pyocin genes, could be explained if MVs are only produced by a small subpopulation that has induced the SOS response. In fact, heterogeneous induction of the SOS response has been reported for cultures of *E. coli*, where about 1% of the population was reported to be induced29. Promoter–reporter eGFP fusions of Ppha and PrecA and genomic transcriptional fusions revealed that under standard (non-inducing) planktonic growth conditions only a small fraction of the cells (<1%) showed strong fluorescence, while a fusion of the constitutive lac promoter to eGFP was expressed in the large majority of cells (Fig. 7f; Supplementary Fig. 7). Hence, even under optimal planktonic growth conditions a small proportion of the cells stochastically induce the SOS response and pyocin expression.

We have found that explosive cell lysis is associated with the production of MVs in interstitial biofilms and planktonic cultures of *P. aeruginosa*. The *Pseudomonas* Quinolone Signal (PQS) has been reported to be crucial for MV production in *P. aeruginosa*30, whereas a number of other studies have found that PQS is not required for MV production in planktonic cultures under stressed conditions.

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**Figure 5** MVs are present within *P. aeruginosa* interstitial biofilms. (a) f3D-SIM of PAK biofilms cultured in the presence of FM1-43FX (white), scale bar, 1 μm. (b) Frequency distribution of diameters of MVs measured in situ in live PAK biofilms \(n = 268\), bin size = 50 nm. (c) f3D-SIM of PAK biofilms cultured in the presence FM1-43FX (blue) and EthHD-2 (red), scale bar, 2 μm. (d) Quantification of MVs in random fields of view (40 μm × 40 μm) of PAO1 \(n = 35\) and PAO1Δlys \(n = 22\) biofilms cultured in the presence of FM1-43FX and imaged with f3D-SIM.
or unstressed conditions\textsuperscript{31–33}. Therefore, to further explore the contribution of PQS to MV biogenesis we analysed MV production in interstitial biofilms of \textit{pqsA} mutants which do not produce PQS\textsuperscript{34}. These assays showed that the \textit{pqsA} mutants of \textit{P. aeruginosa} strains PAO1 and PA14 were not defective in the production of MVs in interstitial biofilms relative to their isogenic parent strains (Fig. 8). In fact PA14\textit{pqsA} produced significantly more MVs than its isogenic parent in this assay. Furthermore, our RNA-seq analyses of MVs obtained from unstressed planktonic cultures revealed that the small RNA PA3305.1 (PhrS), which has been shown to stimulate \textit{Pseudomonas} quinolone signal (PQS) production\textsuperscript{35}, is less abundant in planktonic MVs than planktonic cells. This is consistent with PQS not having a critical role in the production of MVs with RNA cargo which have presumably been derived through explosive cell lysis. These observations indicate that explosive cell lysis-mediated MV production in biofilms and planktonic cultures is independent of PQS.

**Discussion**

In this study, we have shown that explosive cell lysis accounts for the efficient liberation of a variety of cellular components including cytosolic proteins, eDNA and MVs that may serve as public goods in \textit{P. aeruginosa} biofilms. We have determined that explosive cell lysis in \textit{P. aeruginosa} is due to an endolysin-encoding gene (\textit{lys}) which is located on the genome within a cryptic prophage gene cluster that encodes the R- and F-type pyocins. Expression of genes in the R- and F-pyocin gene cluster, including \textit{lys}, is known to be upregulated by exposure to exogenous stresses through the RecA-dependent SOS response\textsuperscript{12,17–21} and we found that explosive cell lysis and \textit{lys} expression is induced by exposure to exogenous stresses and that this is regulated through RecA. However, it is yet to be determined if explosive cell lysis under non-stress conditions is a programmed cell death pathway induced by fratricide or altruistic suicide\textsuperscript{36}. We found that \textit{P. aeruginosa} \textit{ΔrecA} strains were significantly abrogated in explosive cell lysis events even in the absence of exogenous stress, which suggests that there may be endogenous cues that induce RecA-mediated \textit{lys} expression. Endogenous stress that activates RecA is known to occur in \textit{P. aeruginosa} biofilms\textsuperscript{37}. Furthermore, the RecA-mediated SOS stress response can lead to a block in cell division in some bacteria\textsuperscript{38} and we observed that many of the cells that go on to become round cells appeared to be blocked in cell division. Therefore it is possible that endogenous stress may act as a trigger to stimulate Lys-mediated explosive cell lysis. Alternatively, explosive cell lysis in \textit{P. aeruginosa} may be a consequence of stochastic expression of the pyocin gene locus (including \textit{lys}) to enable release of pyocins from the cell. Interestingly, we found that the pyocin structural genes are not required for explosive cell lysis to occur. Therefore, it is possible that \textit{lys} may also be expressed independently of the structural genes of the R- and F-pyocin locus and that explosive cell lysis can occur independently of pyocin release. Whatever the mechanism by which explosive cell lysis is triggered, it is clear from our observations that this process liberates public goods including eDNA, cytosolic content and MVs that can be exploited by other cells. Indeed, we found that populations of \textit{lys} mutants that are unable to undergo explosive cell lysis are severely abrogated in biofilm formation.

Also encoded in the R- and F-pyocin gene cluster is the putative holin, Hol (PA0614)\textsuperscript{17}, overexpression of which has been
shown to cause increased lysis of *P. aeruginosa*[^39]. While we have not examined the contribution of Hol in explosive cell lysis in this study, we expect that Hol facilitates translocation of Lys across the inner membrane to degrade the cell wall peptidoglycan leading to explosive cell lysis. Indeed, here we showed that *in trans* expression of both hol and lys were required to elicit cell lysis in *E. coli* (Supplementary Fig. 2) indicating that Hol facilitates translocation of Lys across the *E. coli* inner membrane.

**Figure 7 | Lys is involved in stress-induced MV formation of planktonic cells.**

(a) MV production in *P. aeruginosa* PAO1 and isogenic mutants were analysed after 16 h of incubation under oxic planktonic growth conditions. *n* = 3; mean ± s.d. (b) MV production in *P. aeruginosa* PAO1 and isogenic mutants were analysed after 16 h of incubation under anoxic planktonic growth conditions. Values indicate the mean ± s.d. of three replicates. *n* = 3; mean ± s.d. #*P* < 0.001 versus wild type (WT) (Student’s *t*-test). (c) MV production by planktonic *P. aeruginosa* PAO1 and isogenic mutants cultured in the presence of MMC (200 ng mL⁻¹) relative to no MMC, *n* = 3; mean ± s.d. #*P* < 0.0005 (Student’s *t*-test). (d) Catalytic activity of Lys is required for genotoxic stress-induced MV formation, *n* = 3; mean ± s.d. #*P* < 0.0005 (Student’s *t*-test). (e) MA plot showing the comparison of mRNA levels associated with MVs with the transcript levels of stationary phase cells. More and less abundant transcripts in MVs are indicated by red and green dots, respectively (*P* value < 0.02). Transcripts from the pyocin gene cluster (PA0610 to PA0648) are circled in black. (f) Promoter activities of recA, hol and lacZ (control) under non-inducing conditions were monitored by the aid of plasmids containing transcriptional fusions of the respective promoter regions to eGFP. Cells expressing GFP are green; scale bar, 2.5 μm.
Figure 8 | PQS is not required for MV production in interstitial biofilms.

(a) EBD-SIM of PA14pqsA and PAO1pqsA biofilms cultured in the presence of FM1-43FX (white) showing MV patches; scale bar, 1 μm. (b) Quantification of MVs in random fields of view (40 μm × 40 μm) of PA14 (n = 54), PA14pqsA (n = 60), PAO1 (n = 22) and PAO1pqsA (n = 24); #P < 0.0001, unpaired t-test with Welch's correction.

However, as other putative P. aeruginosa holins CdA and Apb441 have previously been reported to be associated with lysis of P. aeruginosa cells, it is possible that these may also contribute to translocation of Lys across the inner membrane.

The R- and F-pyocin gene cluster is part of the P. aeruginosa accessory genome as it is not present in all strains42. However, we found that lys is highly conserved in all complete genomes of P. aeruginosa strains available in the public genome databases (EMBL/GenBank/DDJB), with 88–100% nucleotide identity over the full length of the 630 bp gene. It is likely that other prophages also encode the endolytic activity required for explosive cell lysis. Furthermore, genes with high similarity to lys were also found in the genomes of other Pseudomonas species and many other bacterial genera indicating that this might be a conserved phenomenon in bacteria.

Using live-cell super-resolution microscopy, we have determined that explosive cell lysis is a mechanism for bacterial MV biogenesis and MV cargo packaging. We found that the vesicularization of shattered membrane fragments that are produced by exploding bacteria are likely to capture cellular components released into the extracellular milieu as they self-annex into MVs. To our knowledge, this is the first direct observation in live bacterial cells of either MV biogenesis or the process by which cellular content is liberated into the extracellular matrix of bacterial biofilms. Although MV biogenesis via explosive cell lysis is unlikely to control the cargo of MVs precisely, this mechanism provides a convincing explanation as to why cytoplasmic material such as DNA, RNA and cytoplasmic proteins are present in MVs. Interestingly, we found that lys mutants were significantly reduced in MV production in biofilms and in stressed planktonic cultures indicating that most MVs are produced through explosive cell lysis events under these conditions. In contrast, however, we found that lys mutants were not defective in MV production in non-stressed planktonic cultures, which suggests that other mechanisms such as outer membrane blebbing may account for the majority of MV biogenesis under these conditions. However, as our RNA-Seq data was obtained from MVs obtained from non-stressed planktonic cultures and showed upregulation of SOS response genes including recA and lys, it is likely that explosive cell lysis also accounts for the production of some MVs that contain cytoplasmic content (including RNA) in non-stressed planktonic cultures. Indeed, during our submerged biofilm formation assays, we observed explosive cell lysis events in the planktonic phase.

Taken together, our data demonstrate a novel role for bacteriophage-associated endolysins in MV biogenesis. These results not only imply that the ability to produce MVs can be conferred via bacteriophages as a consequence of the bacteriophage lytic cycle but also may explain why MVs can harbour complete viral genomes43 and DNA associated with MVs isolated from open ocean samples are strongly enriched for viral sequences28,44.

As many species of bacteria and archaea produce MVs, moonlighting proteins and/or a biofilm matrix comprised of eDNA, lipids and cytoplasmic proteins4,8,45, and prophage and prophage-like elements are a common feature of bacterial genomes46, phage-mediated explosive cell lysis may be a ubiquitous mechanism for the production of MVs and release of cytosolic public goods in bacterial biofilms. At low levels, phage-mediated explosive cell lysis is likely to be beneficial to bacterial communities through the provision of a mechanism for the efficient release of cell-derived public goods. Indeed, prophage and prophage-like elements have been associated with increased fitness of bacterial populations12 and the endolysin genes of cryptic prophages appear to experience purifying evolution, which suggests that they are under positive selection pressure47.

**Methods**

**Bacterial strains and plasmids.** P. aeruginosa and E. coli strains used in this study, as well as plasmids, are listed in Supplementary Table 1. The primers used in this study are listed in Supplementary Table 2. Gene deletion mutants and genomic eGFP transcriptional fusions were carried out using pG19II (ref. 48) for homologous recombination as previously described49. The pG19II-derived plasmids were inserted into P. aeruginosa strains through conjugation with E. coli S17-1. The mutants were analysed by PCR. PA0629 fused with six histidine residues was cloned into the expression vector pJN105 by using cPA0629H_F/cPA0629H_R primer pairs to amplify this region of the PA01 chromosome. PA0614 was cloned in to the expression vector pET21b by using PA0614b_F/PA0614b_R primer pairs to amplify the PA01 chromosome. PA0629 was constructed by overlap extension PCR by inserting a point mutation that replaced the catalytic glutamic acid (E51) with valine. For the first round PCR, the PA01 chromosome was amplified with PA0629AA_F/PA0629_E51V_R or PA0629_E51V_F/cPA0629H_R primer pairs. The PCR products were mixed for overlap extension PCR50. Finally, the full-length PA0629 was amplified with cPA0629H_F/cPA0629H_R and cloned into pJN105. Point mutation was confirmed by sequencing (Hokkaido System Science, Japan). The catalytic site was predicted by the Phyre server51. The recA promoter region was introduced into pMEGFP, by amplifying this region of the PA01 genome with pRecA_F/pRecA_R primer pairs as previously described52. The lac promoter was amplified from pUC9 with pLac_F/pLac_R primers and inserted into pMEGFP, to construct the constitutive eGFP expression vector, pMlAC-G. The plasmid pUCpCtGFP was constructed by PCR amplifying the mChFP gene from the template pmCherry-C2 (CLONTECH Laboratories, Inc., Palo Alto, CA, USA) using the primer pair mCherry_F/mCherry_R and cloning the expression fragment into the SpiII and HindIII sites of pUCPKS.

**Growth conditions.** P. aeruginosa and E. coli were cultured in either cation-adjusted Mueller Hinton broth (CAMHB) or Luria-Bertani (LB) broth for P. aeruginosa or LB broth for E. coli or on LB agar (1.5%) and incubated at 37°C. Antibiotic concentrations used for selection of E. coli were 100 μg ml⁻¹ ampicillin, 10 μg ml⁻¹ gentamicin and 10 μg ml⁻¹ tetracycline and for P. aeruginosa were 250 μg ml⁻¹ carbencillin and 100 μg ml⁻¹ gentamicin. Fluorescent stains used in this study (obtained from Life Technologies) were the eDNA stain TOTO-1 iodide (1 μM), the lipophilic membrane stain FM1-43FX (5 μg ml⁻¹), and the eDNA and dead cell dye ethidium homodimer-2 (EtHDH-2; 1 μM). DNAase (D5025, Sigma) was used at 100 Kunitz units per ml. For MV assays, P. aeruginosa was grown aerobically at a starting optical density of 0.01 at 600 nm (OD600), unless otherwise specified. For P. aeruginosa anoxic cultures, LB medium was supplemented with 100 mM KNO₃ in butyl-rubber sealed Hungate tubes and the head space was replaced with argon by flushing gas through a needle45. No growth was observed under the anoxic condition, when KNO₃ was not added in the medium or when a nitrate reductase mutant was inoculated, confirming that the growth was dependent on denitrification. MMC and CPIFXL were used to induce MV production at concentrations indicated in the figure legends. L-Arabinose was added at concentrations indicated in the figure legends to induce gene expression under the control of araBAD promoter on pN105.

**Biofilm assays.** Interstitial biofilm assays were performed as described previously53. Briefly, microscale slides were coated in nutrient media solidified with gellan gum (TMGG; 0.4 × LB, 0.1% MgSO₄ · 7H₂O, 0.8% GelGro gellan gum (MP
Biomedicals, Santa Ana, CA, USA) and where indicated fluorescent stains were added to the molten media immediately prior to pouring. Once set, the TMGG slabs were inoculated with 0.5 cm2 of overnight plate culture, a cover-slip applied and incubated at 37 °C for 4–6 h prior to microscopic imaging. Filter disc diffusion assays were performed as described previously25 with filter discs saturated in CPEFLX (100 μg ml–1), MMC (500 μg ml–1) or sterile water. Briefly, 75 μl of the test solution was applied to a filter disc (Whatman 6 mm, GE Healthcare). Each disc was dried for 2 h and then applied to a TMGG coated microscope slide and a gradient allowed to establish for 1 h and the disc removed. The TMGG was inoculated with the strain of interest 5 mm from the disc, a cover-slip applied and incubated at 37 °C for 4 h.

To assay the formation of submerged biofilms, overnight cultures were washed three times in fresh CAMHB, diluted to an equivalent of 1/100 in CAMHB and cultured for 2 h at 37 °C with shaking (250 r.p.m.). The cultures were then transferred to an eight-well IBIDI-treat chamber slide (IBIDI GmbH, Germany) and incubated statically at 37 °C. To examine eDNA release during the initial stages of biofilm formation, the biofilm culture media included the eDNA stain TOTO-1 and time-lapse imaging (DV Elite; ×100 objective) commenced after 1 h static culture. To visualize biofilm formation after 8 h static culture, wells were washed twice with fresh media. CAMHB containing eDNA stain was added to the wells and biofilms and cells at the substratum imaged with phase contrast and wide-field fluorescence microscopy (Olympus IX71, ×100 objective). To assess influence of exogenous eDNA on formation of submerged biofilms, P. aeruginosa PA01 chromosomal DNA was purified (MasterPure DNA purification kit, Epicentre) and added to CAMHB at 1 μg ml–1.

**MV isolation and quantification.** Cell culture was centrifuged for 10 min at 15,000g, 4 °C, and the supernatant filtered through a 0.4 μm pore size polyvinylidene difluoride filter (Merck Millipore, Germany). The supernatant was ultracentrifuged for 1 h at 150,000g, 4 °C, and the pellet resuspended in double distilled water for MV quantification and in Optiprep (AXIS-SHIELD, Scotland) added to the molten media immediately prior to pouring. Once set, the TMGG slab assays were performed as described previously52 with filter discs saturated in Optiprep (AXIS-SHIELD, Scotland) and incubated statically at 37 °C. To examine eDNA release during the initial stages of biofilm formation, the biofilm culture media included the eDNA stain TOTO-1 and time-lapse imaging (DV Elite; ×100 objective) commenced after 1 h static culture. To visualize biofilm formation after 8 h static culture, wells were washed twice with fresh media. CAMHB containing eDNA stain was added to the wells and biofilms and cells at the substratum imaged with phase contrast and wide-field fluorescence microscopy (Olympus IX71, ×100 objective). To assess influence of exogenous eDNA on formation of submerged biofilms, P. aeruginosa PA01 chromosomal DNA was purified (MasterPure DNA purification kit, Epicentre) and added to CAMHB at 1 μg ml–1.

**RNA isolation.** Total RNA from stationary phase PA01 cells and MVs grown aerobically in LB medium was isolated using M-MLV Reverse Transcriptase, RNase H Minus (Promega). Each PCR reaction was run in triplicate containing three dilutions of cDNA (15, 7.5 and 3.75 ng), 12.5 μl of 2 × Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent, Switzerland) and an MX3000P instrument (Agilent). cDNA was synthesized using M-MLV Reverse Transcriptase, RNase H Minus (Promega). Each PCR
sites across the monolayer region of each interstitial biofilm were quantified manually and the area of the biofilm comprised of cells identified by auto-thresholding using Fiji62. To analyse the frequency of microcolonies in submerged biofilms, random images of the substrate surface were obtained (Olympus IX71; ×40 objective) and ‘Particles’ (microcolonies) > 100 μm2 identified by auto-thresholding using Fiji62. Quantitative assessment of cell morphotypes in interstitial biofilms was performed using an in-house program, BacFormatics v0.7 (source code available at https://github.com/ithreeMIF/BacFormatics), that we have developed in MATLAB (The MathWorks Inc., Natick, MA, USA). BacFormatics is based on the open-source TACTICS Toolbox63,64. The following image analysis pipeline in BacFormatics was utilized to analyse 16-bit phase-contrast images. Pixel intensities of each image were inverted (I = 65,535 – I). To segment individual bacteria we integrated an edge-detection algorithm and enhanced the background between the cells. This includes morphological operations to connect and close the spaces between the cells, which has a net-like closed structure and is useful to split touching cells. To detect cell perimeters we used Laplacian of Gaussian (LoG) edge-detection as described previously65. The LoG edge-detection provides closed contour curves, which are filled and represent segmented bacteria. To accurately segment the net, we applied multiple structuring element centre surround top-hat transformation66. Briefly, multiple structuring elements were applied to enhance linear regions at different directions, followed by intensity thresholding to reconstruct the image. A morphological close operation was applied to each image to close spaces in the morphology of the cells (to smooth the edges)67. This step was followed by a background subtraction, which was applied by removing the mean intensity of the background from each pixel in the image. Cells were segmented by intensity threshold, where the cutoff level was manually adjusted or automatically chosen by Otus’s method68. Following the segmentation step, small segments (less than 80 pixels) and large segments (more than 3,000 pixels) were removed. Intensity pixels with zero values within the cells (holes) were converted to values of one. To detect cell clusters, the curves and junctions of the cells were split using an algorithm written by He and Yung69, then the watershed algorithm was applied to separate segments that were classified as touching objects. To split two overlapping cells we trained a data set with 100 touching cells and utilized the MATLAB function classify from the Statistics Toolbox. The classifier is based on morphology parameters and ratio between concave borders at the intersection of two touching cells as shown previously70. Categorization of cells as round or rod morphotypes was based on circularity criteria calculated by the standard formula:

$$F_{circ} = \frac{4 \cdot \pi \cdot A}{p^2}$$

where A is the area and P is the cell perimeter. Cells with a circularity factor >0.8 were labelled as a round morphotype and <0.8 as a rod morphotype. Manual inspection was applied using a dedicated user interface that colour-labelled round cells. BacFormatics requires MATLAB R2012b version 8.0 (The MathWorks, Inc.) or later versions with the presence of the Statistics Toolbox and MATLAB Image Processing Toolbox (IPT). The BacFormatics analysis in this paper was performed on a Dell Latitude E5540 with 16 GB RAM and Intel(R) Core(TM) i7-4600U CPU 2.10GHz.

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