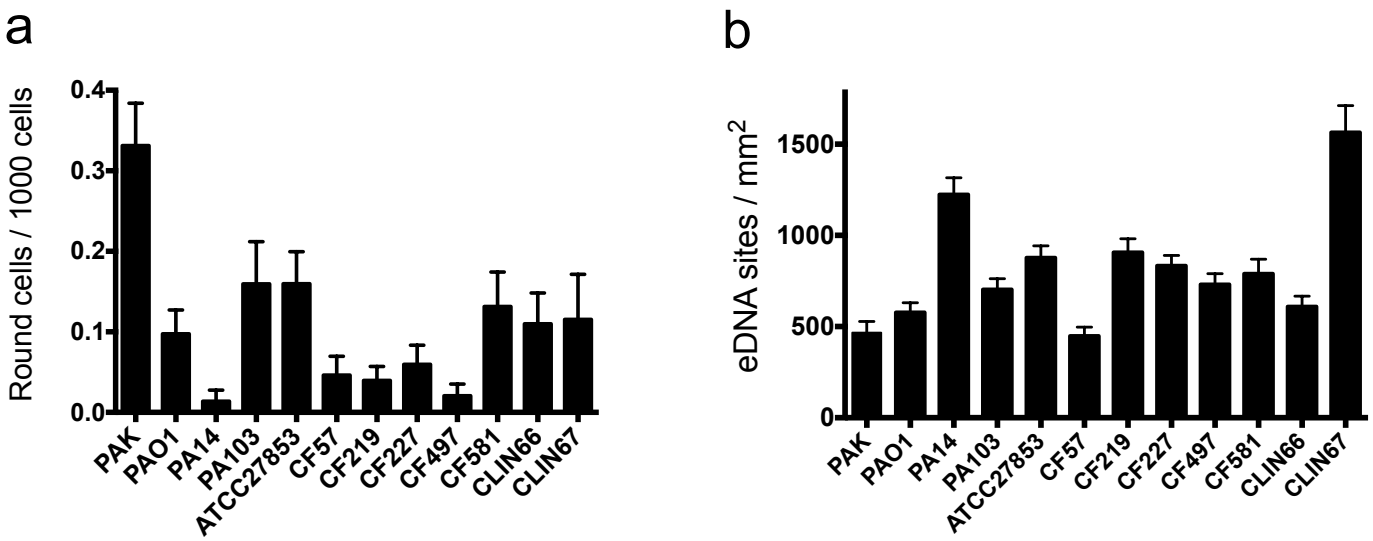
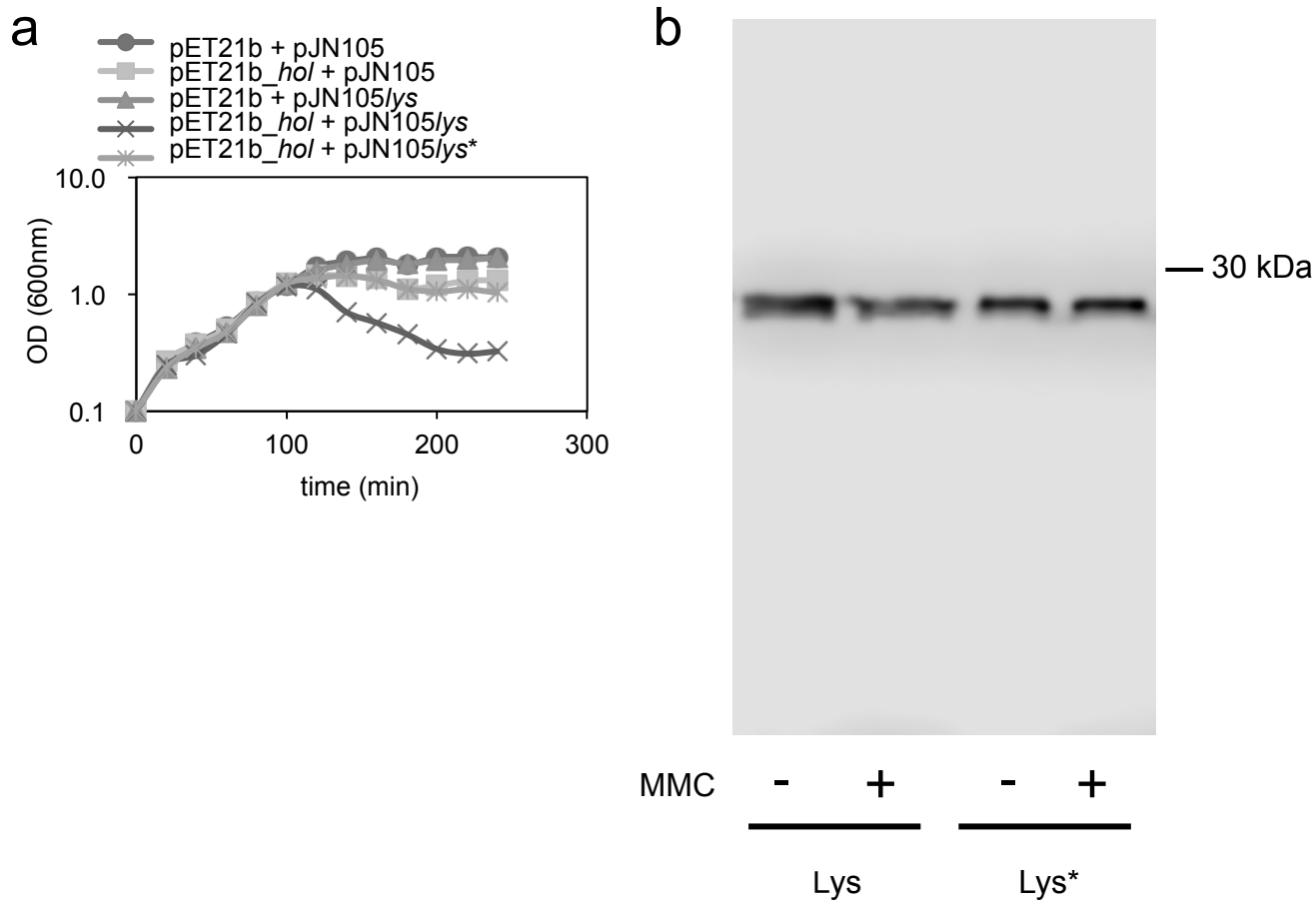
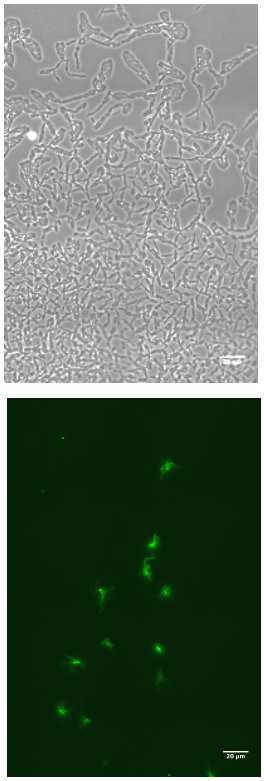



**Supplementary Figure 1. Explosive cell lysis is a conserved phenotype in *P. aeruginosa*** (a) Proportions of cells with round cell morphotypes in interstitial biofilm monolayers of laboratory and clinical *P. aeruginosa* strains. Computer vision was used to identify cells in 60 random images of each strain of *P. aeruginosa* (except PAK, 111 images) and characterize these as having either rod or round morphotypes. The total number of cells detected for each strain was; PAK 161628, PAO1 114559, PA14 118997, PA103 89323, ATCC27853 94866, CF57 122407, CF219 116836, CF227 98526, CF497 97103, CF581 105114, CLIN66 91460, CLIN67 57126. (b) eDNA sites in interstitial biofilms of *P. aeruginosa* strains.




**Supplementary Figure 2. Lys activity is required for cell lysis** (a) Hol, Lys and Lys* were separately or jointly expressed in *E. coli* BL21(DE3). Cell lysis was induced by expressing both Hol (holin) and Lys (endolysin). Lys*, which carries a point mutation in the active site of the enzyme, did not induce cell lysis. Following 1h of incubation, expression of genes was induced by the addition of 0.1mM IPTG and 0.5% arabinose. Representative data of two independent experiments are shown. (b) Lys and Lys* expression levels are indistinguishable. Lys and Lys* were His tagged and their expression levels in the PAO1Δ*lys* mutant background were determined by Western blotting using anti-His antibodies. Samples were taken at the time point when MVs were collected. 2.5 µg of protein was used for SDS-PAGE.

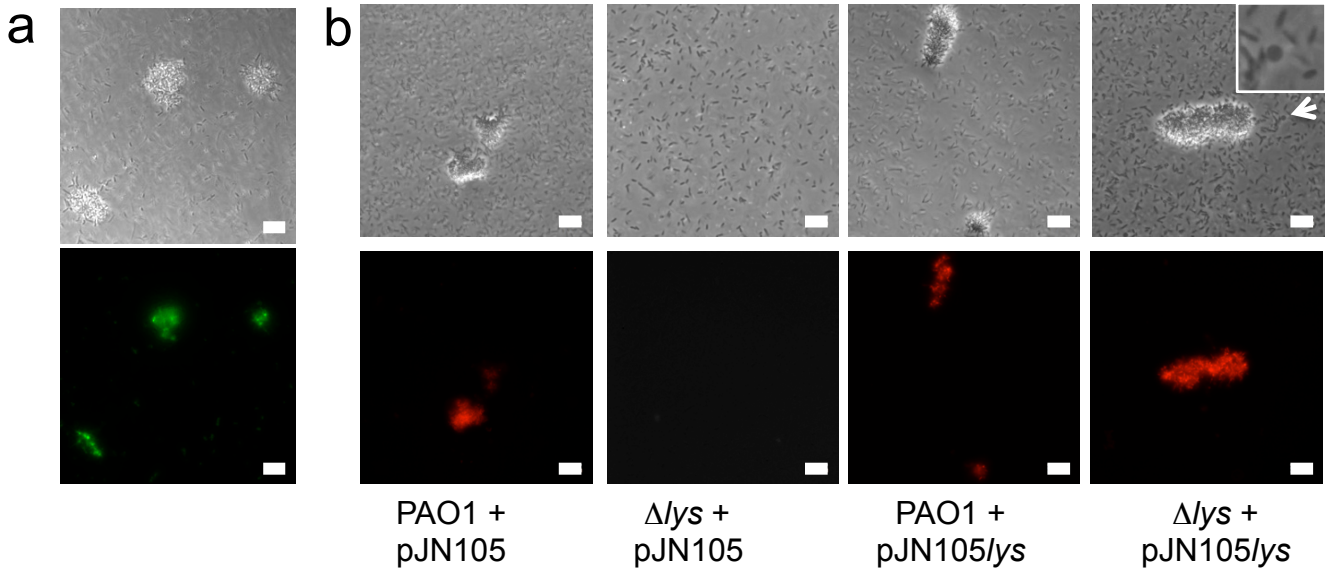
a



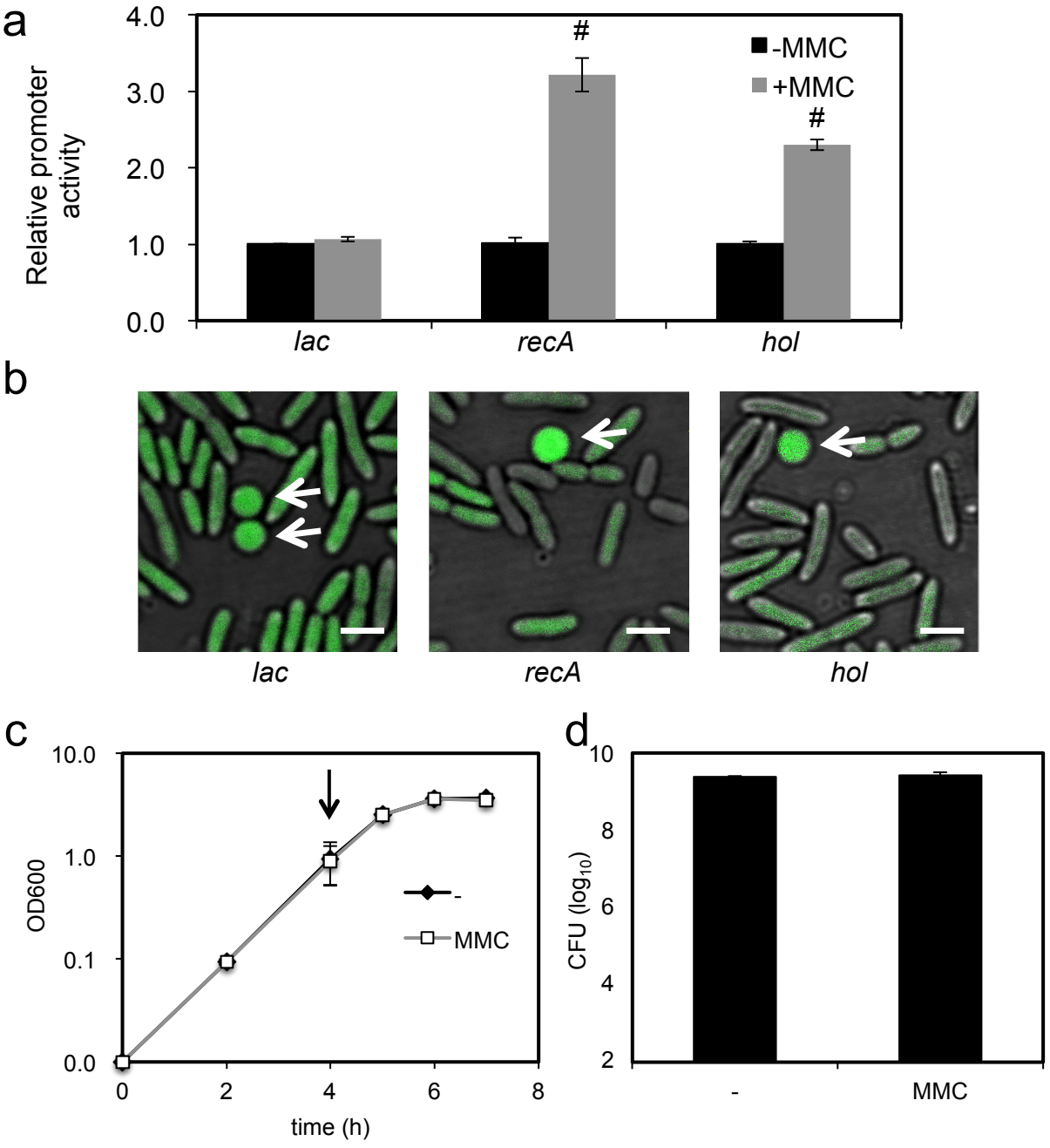
b

| PA14 Gene | PAO1 Ortholog | Description |
|---|---|---|
| PA14_07980 | PA0613 | conserved hypothetical protein |
| PA14_08010 | PA0616 | putative baseplate assembly protein V |
| PA14_08030 | PA0630 | hypothetical protein |
| PA14_08040 | PA0619 | putative phage tail protein |
| PA14_08050 | PA0620 | putative tail fiber protein |
| PA14_08060 | PA0621 | putative tail length determinator protein |
| PA14_08070 | PA0622 | putative tail fiber assembly protein |
| PA14_08090 | PA0623 | putative phage tail tube protein |
| PA14_08100 | PA0640 | putative phage tail assembly protein |
| PA14_08120 | PA0636 | putative tail length determination protein |
| PA14_08130 | PA0618 | putative phage baseplate assembly protein |
| PA14_08180 | PA0615 | conserved hypothetical protein |
| PA14_08210 | PA0633 | putative major tail protein V |
| PA14_08220 | PA0634 | hypothetical protein |
| PA14_08230 | PA0626 | putative tail formation protein |
| PA14_08240 | PA0647 | conserved hypothetical protein |
| PA14_08260 | PA0638 | putative minor tail protein L |
| PA14_08270 | PA0624 | conserved hypothetical protein |
| PA14_08280 | PA0635 | hypothetical protein |
| PA14_08300 | PA0641 | putative phage-related protein, tail component |
| PA14_08330 | PA0648 | hypothetical protein |
| PA14_08320 | PA0639 | conserved hypothetical protein |

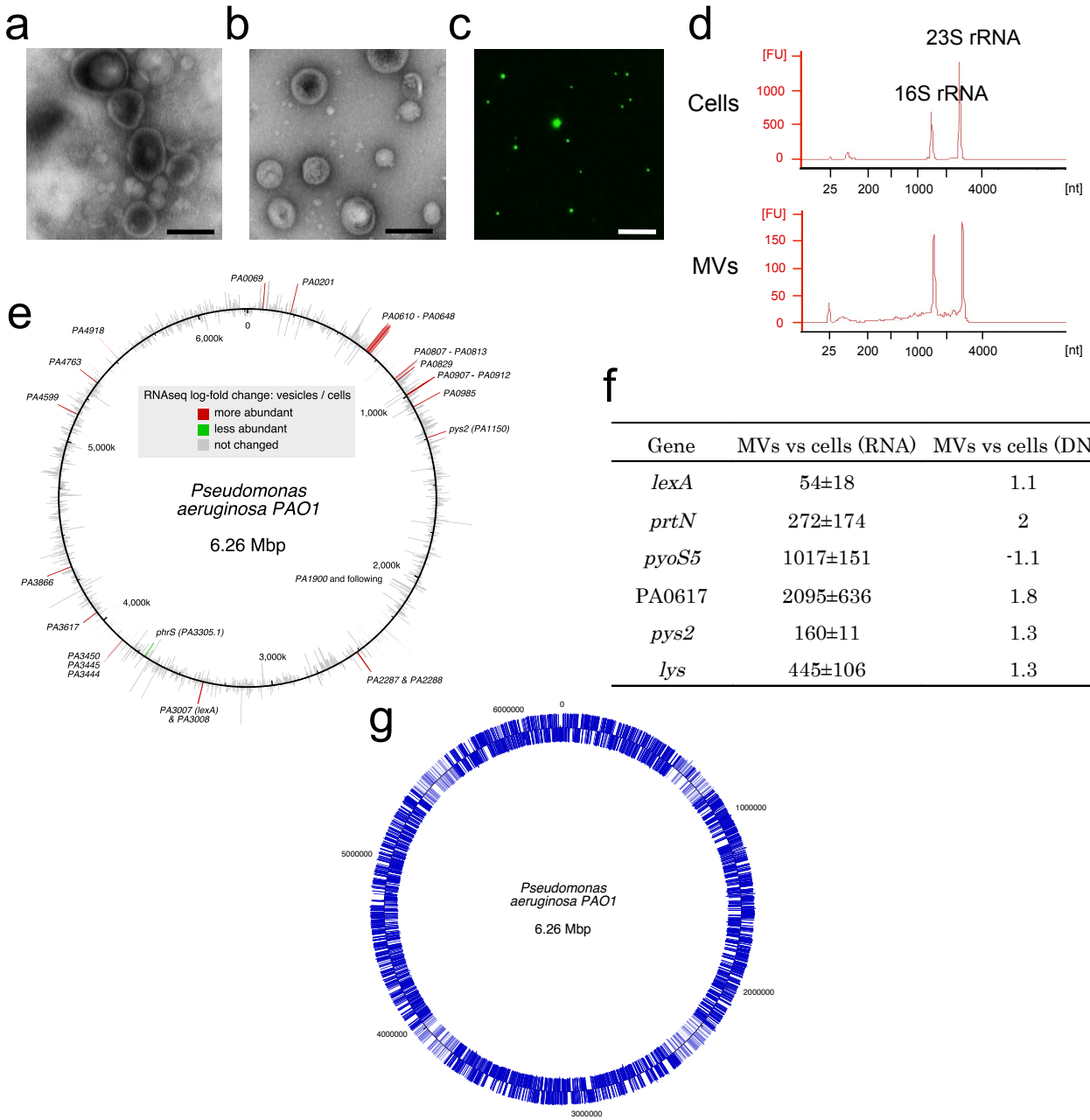
**Supplementary Figure 3. Pyocin structural genes are not required for eDNA release in interstitial biofilms.** (a) Phase-contrast (top) and TOTO-1 stained eDNA (green, bottom) of interstitial biofilms of *P. aeruginosa* strain PA14. (b) Table showing PA14 mutants of pyocin structural genes examined for defects in eDNA release in interstitial biofilms. No defects were identified in any of the mutants indicating that the production of pyocins *per se* is not required for eDNA release through explosive cell lysis.




**Supplementary Figure 4. Lys is required for microcolony formation in submerged biofilms.** (a) Microcolonies in 8 h submerged biofilms of *P. aeruginosa* strain PAO1. Representative phase contrast (top) and eDNA (TOTO-1, bottom) images, scale bar 10 μm. (b) Microcolonies in 8 h submerged biofilms of PAO1 and PAO1Δ*lys* containing vector control (pJN105) or complementation plasmid (pJN105*lys*). Representative phase contrast (top) and eDNA (EthHD-2, bottom) images, scale bar 10 μm. Inset shows magnified view of round cell at arrow-head.

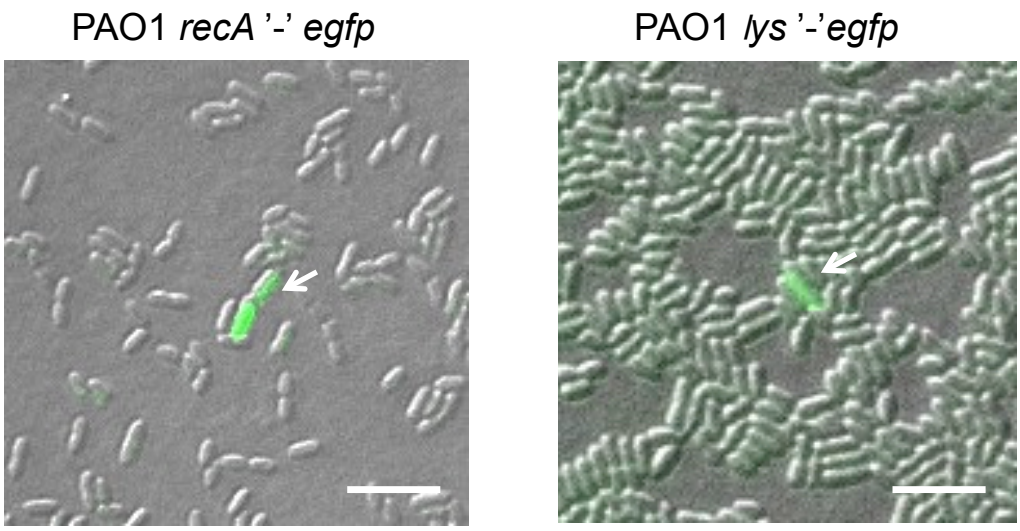



**Supplementary Figure 5. Induction of *recA* and *hol* expression by MMC.** (a) Promoter activities of transcriptional fusions to *eGFP* present on plasmids pMLAC-G, pMRECA-G and pM0614-G in *P. aeruginosa* PAO1 cultures were determined. Data represent relative eGFP fluorescence of cultures treated with 200 ng $mL^{-1}$ MMC normalized against non-treated cultures. Values indicate the mean ± s.d. of three replicates. # $P < 0.0005$ versus MMC non-treated cultures (Student's *t* test). (b) *lacZ*, *recA* and *hol* promoter expression in induced cells. Arrows indicate rounded cells that express *eGFP*. Exponentially grown cells were treated for 1.5 h with MMC (200 ng $mL^{-1}$) in liquid culture, and further incubated for 1.5 h on a 0.5% agarose pad supplemented with LB and MMC (200 ng $mL^{-1}$); scale bar, 2 µm. (c) and (d) Bacterial growth was unaffected when cells were grown in the presence of 200 ng $mL^{-1}$MMC. (c) Growth curves indicating the time points when MMC (200 ng $mL^{-1}$) was added (arrow). (d) CFUs of the cultures after 7h of growth in absence or presence of MMC (200 ng $mL^{-1}$). MMC was added at the same time point as (c). Values indicate the mean ± s.d. of three replicates.




| Gene | MVs vs cells (RNA) | MVs vs cells (DNA) |
|---|---|---|
| *lexA* | 54±18 | 1.1 |
| *prtN* | 272±174 | 2 |
| *pyoS5* | 1017±151 | -1.1 |
| PA0617 | 2095±636 | 1.8 |
| *pys2* | 160±11 | 1.3 |
| *lys* | 445±106 | 1.3 |

**Supplementary Figure 6. DNA and RNA associated with MVs derived from *P. aeruginosa*.** Transmission electron micrograph (TEM) of MVs collected from a stationary phase (a) and a MMC (b) treated *P. aeruginosa* PAO1 culture, scale bar, 100 nm. (c) Epifluorescence micrograph of purified MVs stained with SYTO RNASelect Green, scale bar, 10 µm. (d) The 23S and 16S rRNAs associated with MVs appear to be intact. The quality of the RNA collected from planktonic cells and purified MVs were analyzed with Agilent BioAnalyzer. The large peaks correspond to 16S rRNA and 23S rRNA. (e) Distribution of RNA-Seq reads of transcripts associated with MVs compared with transcripts of planktonic cells. The log-fold-changes of reads per transcript are plotted with respect to genomic location, with transcripts enriched in MVs pointing outwards and transcripts enriched in planktonic cells pointing inwards. More abundant transcripts associated with MVs are indicated in red and the less abundant transcript (*phrS*) is indicated in green. Differential abundance was determined as described in the Methods ("Illumina sequencing of RNA and DNA extracted from MVs"). (f) Table showing validation of the RNA-Seq data by qPCR of RNA isolated from purified MVs. Several transcripts that showed increased abundance in MVs relative to planktonic cells in the RNA-sequencing analysis were selected and their expression levels were validated by qPCR. The *rpoD* transcript was used for data normalization. The corresponding DNA was also analyzed as a control. Values indicate the mean fold-change ± s.d. of three replicates. (g) Distribution of DNA-seq reads of DNA associated with purified MVs. Log(RPKM) values are plotted with respect to genomic location, with the + strand on the outside and the - strand on the inside.




**Supplementary Figure 7. Heterogeneous expression of SOS-regulated genes in a transcriptional single copy *eGFP* fusion strain.** *eGFP* was transcriptionally fused to the 3′-end of *recA* and *lys* and integrated as single copy in the genome of *P. aeruginosa* PAO1. Planktonic cells were cultured under non-inducing conditions and examined with fluorescence microscopy. Arrow indicates cells with high levels of GFP expression showing heterogenous expression of *recA* and *lys*. These singly copy transcriptional fusions confirm the results obtained with the plasmid-based reporters shown in Fig. 7. However, the plasmid-born fusions gave rise to a much stronger fluorescent signal than the respective chromosomal fusions, scale bar, 5µm.

# Supplementary Table 1. Strains and plasmids used in this study.

| Strain, plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| **Strains** | | |
| ***E. coli*** | | |
| K12 BW25113 | *lacI*$^q$, rrnB$_{T14}$, Δ*lacZ*$_{WJ16}$, *hsdR*514, Δ*araBAD*$_{AH33}$, Δ*rhaBAD*$_{LD78}$ | Keio Library |
| DH5α | *E. coli* strain for transformation (F$^-$, *lacZ*ΔM1, *recA1*) | TaKaRa |
| S17-1 | Mobilizer strain | 1 |
| BL21(DE3) | F$^-$, *ompT*, *hsdS*$_B$ (r$_B^-$m$_B^-$), *gal*, *dcm*, (DE3) | TaKaRa |
| ***P. aeruginosa*** | | |
| PAK | Wildtype | John Mattick, University of Queensland |
| PA14 | Wildtype | Frederick Ausubel, Harvard University |
| PAO1 | Wildtype also referred to as MPAO1 | Colin Manoil, University of Washington |
| PAO1_Nott | Wildtype isogenic parent strain of PAO1*pqsA* | Paul Williams, University of Nottingham |
| PA103 | Wildtype | ATCC |
| ATTC27853 | Wildtype | ATCC |
| CF57 | CF isolate | David Armstrong, Monash Children's Hospital |
| CF219 | CF isolate | David Armstrong, Monash Children's Hospital |
| CF227 | CF isolate | David Armstrong, Monash Children's Hospital |
| CF497 | CF isolate | David Armstrong, Monash Children's Hospital |
| CF581 | CF isolate | David Armstrong, Monash Children's Hospital |
| CLIN66 | Endotracheal aspirate | Peter Midolo, Monash Medical Centre |
| CLIN67 | Chest fluid | Peter Midolo, Monash Medical Centre |
| PAO1Δ*recA* | *recA* deletion mutant of PAO1 | This study |
| PAO1Δ*lys* | PA0629 deletion mutant of PAO1 | This study |
| PAKΔ*lys* | PA0629 deletion mutant of PAK | This study |
| PAO1ΔPA0620 | PA0620 deletion mutant of PAO1 | This study |
| PAO1ΔPA0622 | PA0622 deletion mutant of PAO1 | This study |
| PAO1ΔPA0641 | PA0641 deletion mutant of PAO1 | This study |
| PAO1::*recA*'-'*eGFP* | Genomic transcriptional fusion of *eGFP* to *recA* | This study |
| PAO1::*lys*'-'*eGFP* | Genomic transcriptional fusion of *eGFP* to PA0629 | This study |
| PAO1*pqsA* | *pqsA* deletion mutant of PAO1_Nott | 2 |
| PA14_51430 | PA14_51430::MAR2xT7; *pqsA* | 3 |
| PA14_08040 | PA14_08040::MAR2xT7; putative phage tail protein | 3 |
| PA14_08050 | PA14_08050::MAR2xT7; putative tail fiber protein | 3 |
| PA14_08300 | PA14_08300::MAR2xT7; putative phage-related protein, tail component | 3 |
| PA14_08090 | PA14_08090::MAR2xT7; putative phage tail tube protein | 3 |
| PA14_08210 | PA14_08210::MAR2xT7; putative major tail protein V | 3 |
| PA14_08260 | PA14_08260::MAR2xT7; putative minor tail protein L | 3 |
| PA14_08220 | PA14_08220::MAR2xT7; hypothetical protein | 3 |
| PA14_08010 | PA14_08010::MAR2xT7; putative baseplate assembly protein V | 3 |
| PA14_08330 | PA14_08330::MAR2xT7; hypothetical protein | 3 |
| PA14_07980 | PA14_07980::MAR2xT7; conserved hypothetical protein | 3 |
| PA14_08180 | PA14_08180::MAR2xT7; conserved hypothetical protein | 3 |
| PA14_08030 | PA14_08030::MAR2xT7; hypothetical protein | 3 |
| PA14_08130 | PA14_08130::MAR2xT7; putative phage baseplate assembly protein | 3 |
| PA14_08230 | PA14_08230::MAR2xT7; putative tail formation protein | 3 |
| PA14_08280 | PA14_08280::MAR2xT7; hypothetical protein | 3 |
| PA14_08100 | PA14_08100::MAR2xT7; putative phage tail assembly protein | 3 |
| PA14_08270 | c PA14_08270::MAR2xT7; conserved hypothetical protein | 3 |
| PA14_08320 | PA14_0832::MAR2xT7; conserved hypothetical protein | 3 |
| PA14_08240 | PA14_08240::MAR2xT7; conserved hypothetical protein | 3 |
| PA14_08120 | PA14_08120::MAR2xT7; putative tail length determinator protein | 3 |
| PA14_08060 | PA14_08060::MAR2xT7; putative tail length determinator protein | 3 |
| PA14_08070 | PA14_08070::MAR2xT7; putative tail fiber assembly protein | 3 |
| | | |
| **Plasmids** | | |
| pUCPSK | *E. coli-Pseudomonas* shuttle vector, Ap$^R$ | 4 |
| pUCPKS | *E. coli-Pseudomonas* shuttle vector, Ap$^R$ | 4 |
| pmCherry-C2 | Source of mCherry fluorescent protein gene (*mCHFP*) | CLONTECH |
| pUCPCFP | *ecfp* sub-cloned into pUCPSK | 5 |
| pUCPmChFP | *mChFP* from pmCherry-C2 sub-cloned into pUCPKS | This study |
| pUC19 | Cloning vector, Ap$^r$ | TakaRa |
| pJN105 | Broad host range arabinose inducible gene expression vector | 6 |
| pET21b | Expression vector, Ap$^r$ | Novagen |
| pJN105*lys* | Arabinose inducible Lys-His expression vector | This study |
| pJN105*lys** | Arabinose inducible Lys*-His expression vector, Lys catalytic site mutated | This study |
| pET21b_*hol* | Hol-His expression vector | This study |
| pG19II | pK19mobsac derived suicide vector; *sacB* Gm$^r$ | 7 |
| pG19recA | *recA* deletion cassette in pG19II | 8 |
| pG19PA0629 | PA0629 deletion cassette in PG19II | This study |
| pG19PA0620 | PA0620 deletion cassette in PG19II | This study |
| pG19PA0622 | PA0622 deletion cassette in PG19II | This study |
| pG19PA0641 | PA0641 deletion cassette in PG19II | This study |
| pG19_*recA*'-'eGFP | *recA'-'eGFP* transcriptional fusion cassette in pG19II | This study |
| pG19_*lys*'-'eGFP | PA0629'-'*eGFP* transcriptional fusion cassette in pG19II | This study |
| pEGFP | Plasmid harboring *eGFP* | CLONTECH |
| pMEXGFP | pMEX9 derived promoter-probe vector; *eGFP,* Gm$^r$ | 8 |
| pMLAC-G | *lac* promoter region fused to *eGFP* in pMEXGFP | This study |
| pMRECA-G | *recA* promoter region fused to *eGFP* in pMEXGFP | This study |
| pM0614-G | *hol* promoter region fused to *eGFP* in pMEXGFP | 8 |

## Supplementary Table 2. Primers used in this study.

| Primers | Sequence 5 '→ 3' (restriction enzyme sites are underlined) |
|---|---|
| **Gene deletion** | |
| ΔPA0620_F1 | CCTAAGCTTGGGCTGCGAAGTGCTGATCAGCGTGCTCG |
| ΔPA0620_R1 | CGGGATCCGGGCAGGCCACCGTATTTCGGAGTATTGGTCG |
| ΔPA0620_F2 | CGGGATCCGAGGTGATTCGCAATGGCTACTTTGCTCAGGC |
| ΔPA0620_R2 | GCTCTAGAGGCTGGGCATTGAAGCGACTCTTGCCGTCG |
| ΔPA0622_F1 | CCTAAGCTTGGGCCACCATCCGCACATAACCATGACGTCG |
| ΔPA0622_R1 | CGGGATCCGTAGGTAGATCTCCATTAATGAAAAACCCCGCACG |
| ΔPA0622_F2 | CGGGATCCGGCAGTGGCTCACCGAAGTTCTGGATGTCGC |
| ΔPA0622_R2 | GCTCTAGAGGCGGTTCGTCTCGTCTGTTATCGATGTCCG |
| ΔPA0629_F1 | CCCAAGCTTGGTGCAACCGAGTTTCCGTATCGTTGCCGACG |
| ΔPA0629_R1 | CGGGATCCGGCGATCCTCCTGCACTCCGATGGGTTTCAG |
| ΔPA0629_F2 | CGGGATCCTGCGGTCACGGGCTCAACGAGCTGGC |
| ΔPA0629_R2 | GCTCTAGAGTGTTGAACGAGGTCACGCCTTCGATCTCCAG |
| ΔPA0641_F1 | GCAACTGCAGCTGGCATCTACCTGGGCAATGACTGGCG |
| ΔPA0641_R1 | GCTCTAGAGGTCGTGATGGTCTTGTTCATGACGTTCCTTCAGGC |
| ΔPA0641_F2 | GCTCTAGAGGAGCAGGCCAGGTCAGAGTTGAGATGGG |
| ΔPA0641_R2 | CGGGATCCGGCGCTTCGGAATTCAGCGTGACGGAAACCG |
| ***eGFP* genomic fusion** | |
| recAG_F1 | GGAATTCCGTCGAGATCTACGGTCCGGAATCC |
| recAG_R1 | CGCCTCGAGTCAATCGGCTTCGGCGTCAGCC |
| recAG_F2 | GACTAGTGGAGGCCAATGGCGATCGTGCTCGATAC |
| recAG_R2 | GCTCTAGACGCCCAGTTCTACCGCAACTTCCAC |
| 0629G_F1 | GGAATTCAGCCTGCTGCTCCAAGGCTTCAAGG |
| 0629G_R1 | CGCCTCGAGTCATACAGCACCGCCCTGGCC |
| 0629G_F2 | GACTAGTGGAGGTTCCATGAGCCGGCTCGCTCTGCTCC |
| 0629G_R2 | GCTCTAGACCCGAGCCGTTGTCGGATGCAAAC |
| egfp_F2 | CGCCTCGAGGGAGGTCCATGGTGAGCAAGGGCGAGGAGCTGTTC |
| egfp_R2 | GACTAGTTTACTTGTACAGCTCGTCCATGCCGAGAG |
| **Promoter assay** | |
| pLac_F | CGGAATTCTGTTCTTTCCTGCGTTATCC |
| pLac_R | CGGGATCCGCTGTTTCCTGTGTGAAATTG |
| pRecA_F | CGGGATCCGGAAGTGGTCGAGGCCATGGTGC |
| pRecA_R | CGCAAGCTTCGAGGATTCCGGACCGTAGATCTCG |
| **Expression plasmids** | |
| PA0629AA_F | GAGGTCAAGAGCGTCGAGTTGAAGAACC |
| PA0629_E51V_F | GTCGGCCACGTAAGCAGCCAGTTGAC |
| PA0629-E51V_R | CCGGGTCAACTGGCTGCTTACGTG |
| cPA0629H_F | CTGAATTCCTGAAACCCATCGGAGTGCAGGAGGATCG |
| cPA0629H_R | CCTCTAGATCAGTGGTGGTGGTGGTGGTGTGACAGCACCGCCCTGGC |
| PA0614b_F | GATTCGACATATGAAGCACCGGAACCCGGCCCTG |
| PA0614b_R | CCGCTCGAGATGCGGGCCACGGTCGTCCG |
| mCherry_F | ATAGCATGCTGAGCAAGGGCGAGG |
| mCherry_R | CGCAAGCTTACTTGTACAGCTCGTCC |
| | |
| **qPCR primers** | |
| prtN (PA0610)_F | ATTGGTCTACCGCATCTTCG |
| prtN (PA0610)_R | TGCATGGCCTTGTGACTATC |
| lexA (PA3007)_F | AAGCCGAGATCCTCTCCTTC |
| lexA (PA3007)_R | CCGGAGTCATTTCGATGG |
| pyoS5 (PA0985)_F | AACTGGAGCGGGACTACAGA |
| pyoS5 (PA0985)_R | TGCTTGCGTAACCAGTCTTG |
| PA0617_F | GGCCTGGCTCATCTTAAACA |
| PA0617_R | TTTCCAGCCTTCGCTCAC |
| pys2 (PA1150)_F | ACGGCTTCAGACTTTCCTCA |
| pys2 (PA1150)_R | AGATAGCGATTTGCGCCTTA |
| PA0614_F | CGCTCTGGGTACTGATCCTG |
| PA0614_R | CGTTCGTAGAGACCGACTGC |
| PA0629_F | GACGAGAGGGAGATCGACAC |
| PA0629_R | GTAGGTGTTGTCGGCAATCG |
| PA0576_F | CATCGCCAAGAAGTACACCA |
| PA0576_R | GCGACGGTATTCGAACTTGT |

**Supplementary References**


1 Simon, R., O'Connell, M., Labes, M. & Pühler, A. Plasmid vector for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. *Methods Enzymol* **118**, 640-659 (1986).

2 Aendekerk, S. *et al.* The MexGHI-OpmD multidrug efflux pump controls growth, antibiotic susceptibility and virulence in *Pseudomonas aeruginosa* via 4-quinolone-dependent cell-to-cell communication. *Microbiology* **151**, 1113-1125 (2005).

3 Liberati, N. T. *et al.* An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci USA* **103**, 2833-2838 (2006).

4 Watson, A. A., Alm, R. A. & Mattick, J. S. Construction of improved vectors for protein production in *Pseudomonas aeruginosa*. *Gene* **172**, 163-164 (1996).

5 Gloag, E. S. *et al.* Self-organization of bacterial biofilms is facilitated by extracellular DNA. *Proc Natl Acad Sci USA* **110**, 11541-11546 (2013).

6 Newman, J. R. & Fuqua, C. Broad-host-range expression vectors that carry the L-arabinose-inducible *Escherichia coli araBAD* promoter and the *araC* regulator. *Gene* **227**, 197-203 (1999).

7 Maseda, H. *et al.* Enhancement of the *mexAB-oprM* efflux pump expression by a quorum-sensing autoinducer and its cancellation by a regulator, MexT, of the *mexEF-oprN* efflux pump operon in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **48**, 1320-1328 (2004).

8 Toyofuku, M. *et al.* Membrane vesicle formation is associated with pyocin production under denitrifying conditions in *Pseudomonas aeruginosa* PAO1. *Environ Microb* **16**, 2927-2938 (2014).