Heterogeneous efflux pump expression underpins phenotypic resistance to antimicrobial peptides

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Antimicrobial resistance threatens the viability of modern medical interventions. There is a dire need of developing novel approaches to counter resistance mechanisms employed by starved or slow-growing pathogens that are refractory to conventional antimicrobial therapies. Antimicrobial peptides have been advocated as potential therapeutic solutions due to low levels of genetic resistance observed in bacteria against these compounds. However, here we show that subpopulations of stationary phase *Escherichia coli* and *Pseudomonas aeruginosa* survive tachyplesin treatment without genetic mutations. These phenotypic variants induce efflux, outer membrane vesicles secretion and membrane modifications in response to tachyplesin exposure, sequestering the peptide in their membranes where it cannot exert its antimicrobial activity. We discovered that formation of these phenotypic variants could be prevented by administering tachyplesin in combination with sertraline, a clinically used antidepressant, suggesting a novel approach for combatting antimicrobial-refractory stationary phase bacteria.

Introduction

Antimicrobial resistance (AMR) was estimated to claim 1.27 million lives in 2019 [1] and is predicted to claim 10 million lives annually by 2050 [2]. Besides the deaths caused directly by infections, AMR also threatens the viability of many modern medical interventions such as surgery and organ transplant. This challenging situation has motivated the development of strategies to combat resistance mechanisms utilised by bacterial pathogens, particularly when they enter states of dormancy or slow growth and become more refractory to conventional antimicrobial treatments [3]. In recent decades, natural defences such as bacteriocin proteins, peptides, endolysins, and antibodies have garnered substantial attention as potential clinical antimicrobial agents [4]. Antimicrobial peptides (AMPs) have been advocated as potential therapeutic solutions to the AMR crisis. Notably, polymyxin-B and -E are in clinical use, and additional AMPs are undergoing clinical trials [5]. AMPs can simultaneously target different cellular subsystems: they target the double membrane of Gram-negative bacteria by interacting with negatively charged membrane lipids while simultaneously disrupting cytoplasmic processes such as protein synthesis, DNA replication, cell wall biosynthesis, metabolism, and cell division [6].

Bacteria have developed genetic resistance to AMPs, including proteolysis by proteases, modifications in membrane charge and fluidity to reduce affinity, and extrusion by AMP transporters. However, compared to small molecule antimicrobials, AMP resistance genes typically confer smaller increases in resistance, with polymyxin-B being a notable exception [7,8]. Moreover, mobile resistance genes against AMPs are relatively rare and horizontal acquisition of AMP resistance is hindered by phylogenetic barriers owing to functional incompatibility with the new host bacteria [9], again with plasmid-transmitted polymyxin resistance being a notable exception [10]. However, whether bacterial populations harbour subpopulations that are transiently phenotypically resistant to AMPs without the acquisition of genetic mutations remains an open question. While there is limited understanding regarding AMPs and transient phenotypic resistance, this phenomenon has been widely investigated in the case of small molecule antimicrobials and bacteriophages [11–15]. Phenotypic resistance to antimicrobials is known to result in negative clinical outcomes and contributes to the emergence of genetic resistance [16–19]. Therefore, further investigation into understanding the initial trajectories of the emergence of AMP resistance is required for the development of affordable, safe and effective AMP treatments while simultaneously circumventing the pitfalls that have fuelled the current AMR crisis [20,21].

The cationic β-hairpin AMP tachyplesin [22] is a promising candidate AMP because it displays broad-spectrum, potent antibacterial and antifungal efficacy, minimal haemolytic effect, and minimal evolution of bacterial resistance [8]. It has been speculated that tachyplesin simultaneously targets multiple cellular components [23,24]. However, further investigation is needed to fully understand this aspect, along with exploring bacteria's potential to transiently survive tachyplesin exposure without genetic mutations. Understanding the emergence of phenotypic resistance to tachyplesin could provide valuable insights into optimising the therapeutic potential of tachyplesin against microbial threats.

Achieving inhibitory concentrations proximal to its cellular target is crucial for tachyplesin (and antimicrobials in general) efficacy [25]. At low concentrations, AMPs induce the formation of transient pores in bacterial membranes that allow transmembrane conduction of ions but not leakage of intracellular molecules [5,6]. Beyond a critical ratio between the AMP and membrane lipid concentrations, these pores become stable, leading to leakage of cellular content, loss of transmembrane potential, and eventual cell death [5,6]. Moreover, it is conceivable that

tachyplesin, and other AMPs, must penetrate the bacterial inner membrane and accumulate at intracellular concentrations sufficient to interfere with cellular targets [26]. Simultaneously, tachyplesin must avoid efflux via protein pumps, such as the *Escherichia coli* and *Yersinia pestis* tripartite pumps AcrAB-TolC and EmrAB-TolC, that confer genetic resistance against AMPs like protamine and polymyxin-B [27,28].

Crucially, recent discoveries have unveiled an association between reduced antimicrobial accumulation and transient phenotypic resistance to antimicrobials [29–33]. Therefore, we hypothesise that phenotypic variations in the mechanisms regulating membrane lipid and protein compositions allow bacterial subpopulations to shift the balance between influx and efflux, reduce the intracellular accumulation of tachyplesin and survive treatment without the emergence of genetic mutations.

To test this hypothesis, we investigated the accumulation and efficacy of tachyplesin-1 against individual *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus* cells in their stationary phase of growth. To determine the mechanisms underpinning phenotypic resistance to tachyplesin, we performed comparative gene expression profiling and membrane lipid abundance analysis between subpopulations susceptible and transiently resistant to tachyplesin treatment. We used this knowledge to design a novel combination therapy that increases tachyplesin efficacy in killing non-growing bacteria that are notoriously more refractory to treatment with AMPs or other antimicrobials. Our data demonstrate that streamlining antibacterial drug discovery could be facilitated by addressing the issue of low intracellular drug accumulation. We propose new combination treatments as a promising foundation for developing peptide-based drug combinations against dormant or slow growing bacteria.

Results

Tachyplesin accumulates heterogeneously within clonal *E. coli* and *P. aeruginosa* populations

To investigate the accumulation of tachyplesin in individual bacteria, we utilised a fluorescent derivative of the antimicrobial peptide, tachyplesin-1-nitrobenzoxadiazole (tachyplesin-NBD) [34] in combination with a flow cytometry assay adapted from previously reported protocols [35,36] (Figure S1A-C and Methods).

As expected, we found that bacterial fluorescence, due to accumulation of tachyplesin-NBD in individual stationary phase *E. coli* BW25113 cells, increased an order of magnitude when the extracellular concentration of tachyplesin-NBD increased from 0 to 8 µg mL^{-1}. At the latter concentration, the single-cell fluorescence distribution displayed a median value of 2,400 a.u. after 60 min treatment at 37 °C (Figure 1A). However, a further increase in the extracellular concentration of tachyplesin-NBD to 16 µg mL^{-1} revealed a noticeable bimodal distribution in single-cell fluorescence. We classified bacteria in the distribution with a median value of 2,400 a.u. as low accumulators and those in the distribution with a median value of 28,000 a.u. as high accumulators (blue and red shaded areas, respectively, in Figure 1A and Figure S1D-J).

A further increase in the extracellular concentration of tachyplesin-NBD to 46 µg mL^{-1} still yielded a bimodal distribution in single-cell fluorescence, 43% of the population being low accumulators; in contrast, only 5% of the population were low accumulators when tachyplesin-NBD treatment at 46 µg mL^{-1} was carried out against exponential phase *E. coli* (Figure S2). Notably, the minimum inhibitory concentration (MIC) of tachyplesin-NBD against exponential

phase *E. coli* is 1 µg mL^{-1} [33]. Therefore, our data suggests that even at extracellular concentrations surpassing growth-inhibitory levels, *E. coli* populations harbour two distinct phenotypes with differing physiological states that respond to treatment differently with high accumulators displaying over 10-fold greater fluorescence than low accumulators.

Subsequently, we tested whether this newly observed bimodal distribution of tachyplesin-NBD accumulation was unique to the *E. coli* BW25113 strain. We found that the bimodal distribution in single-cell fluorescence was also present in nine *E. coli* clinical isolates (treated with tachyplesin-NBD at 46 µg mL^{-1} for 60 min) characterised by a diverse genetic background and various antimicrobial resistance genes, such as the polymyxin-E resistance gene *mcr1* [37] (Figure 1B, 1C and Data Set S1). The low accumulator median in strains JD1147 and MC04960 were shifted to the left and to the right, respectively, compared to BW25113, whereas the high accumulator median in the distribution for strain JS1060 was shifted to the left compared to BW25113. Remarkably, all isolates tested harboured low accumulators of tachyplesin-NBD.

Furthermore, to examine whether the bimodal distribution of tachyplesin-NBD accumulation also occurred in other highly virulent bacterial pathogens, we treated three members of the ESKAPE pathogens with 46 µg mL^{-1} tachyplesin-NBD for 60 min while in their stationary phase of growth. In *P. aeruginosa* populations, we observed a bimodal distribution of tachyplesin-NBD accumulation with the presence of low accumulators (Figure 1D). In contrast, we did not find evidence of low tachyplesin-NBD accumulators in *K. pneumoniae* (Figure 1E) and recorded only low levels of tachyplesin-NBD accumulation in *S. aureus* (Figure 1F) in accordance with previous reports [38]. Finally, we tested whether this newly observed bimodal distribution was a feature unique to tachyplesin or is widespread across different AMPs. We found that *E. coli* BW25113 displayed only low accumulators of another β-hairpin AMP, arenicin-NBD, and only high accumulators of cyclic lipopeptide AMPs, polymyxin-B-NBD and octapeptin-NBD (Figure S3).

These data demonstrate that although only limited evolution of genetic resistance against tachyplesin has been previously observed [8], two key pathogens display bimodal accumulation of tachyplesin, but not towards other AMPs against which they develop genetic resistance [8].

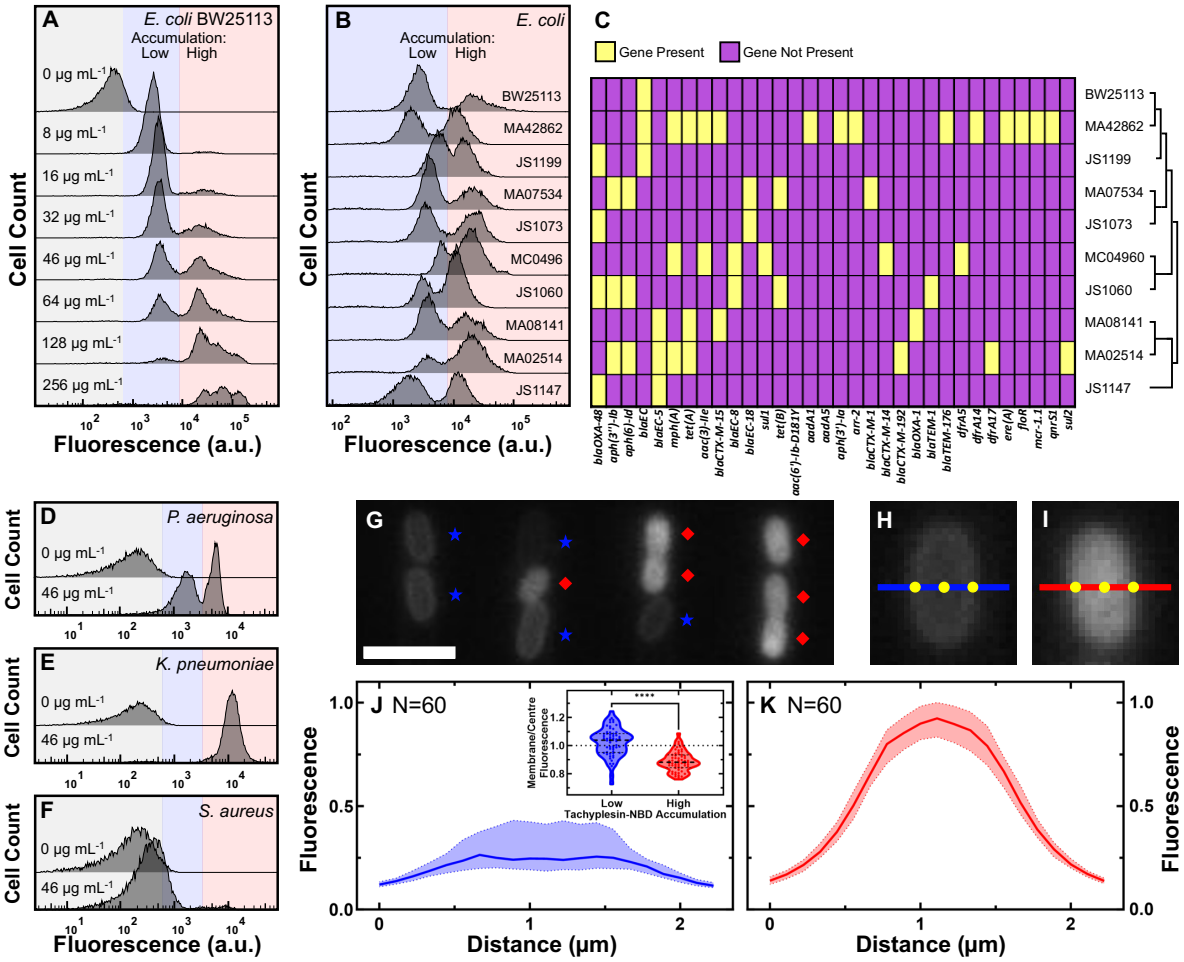

Figure 1. Tachyplesin accumulates heterogeneously within clonal *E. coli* and *P. aeruginosa* populations. (A) Tachyplesin-NBD accumulation in stationary phase *E. coli* BW25113 treated with increasing concentrations of tachyplesin-NBD for 60 min. Shaded regions show low (blue) and high (red) tachyplesin-NBD accumulation within an isogenic *E. coli* BW25113 population. Each histogram reports 10,000 recorded events and it is a representative example of accumulation data from three independent biological replicates (Figure S1D-J). (B) Tachyplesin-NBD accumulation in nine stationary phase *E. coli* clinical isolates treated with 46 µg mL^{-1} tachyplesin-NBD for 60 min. Accumulation data for *E. coli* BW25113 are reproduced from panel A. (C) Heatmap showing the presence of antimicrobial resistance genes and phylogenetic data of the clinical isolates. Corresponding gene products and the antimicrobials these genes confer resistance to are reported in Data Set S1. (D-F) Tachyplesin-NBD accumulation in *P. aeruginosa*, *K. pneumoniae* and *S. aureus* treated with 0 or 46 µg mL^{-1} tachyplesin-NBD for 60 min. Each histogram in each panel shows a representative example of accumulation data from three independent biological replicates. (G) Representative fluorescent images depicting low and high tachyplesin-NBD accumulators within an *E. coli* BW25113 population. Bacteria were continuously exposed to 46 µg mL^{-1} tachyplesin-NBD for 60 min in a microfluidic mother machine device. Blue stars and red diamonds indicate low and high accumulators, respectively. Scale bar: 3 µm. (H and I) Representative fluorescent images of individual low and high tachyplesin-NBD accumulators, respectively. Blue and red lines show a 2.2 µm-long cross-sectional line used for measuring fluorescence profile values in J and K, with the origin on the left side. (J and K) Normalised median (solid line), lower and upper quartiles (dotted lines) of fluorescence profile values of *E. coli* BW25113 cells plotted against the distance along the left side origin of a 2.2 µm-long straight line in low accumulators (J) and high accumulators (K), respectively. Phenotype assignment was further verified via propidium iodide staining (see Figure 2G). Inset: each dot represents the corresponding membrane to cell centre fluorescence ratio measured at the points indicated in H and I, dashed lines indicate the median and quartiles of each distribution. Statistical significance was assessed using an unpaired nonparametric Mann-Whitney U test with a two-tailed P-value and confidence level at 95%. ****: $p<0.0001$. Data was collected from three independent biological replicates.

Tachyplesin accumulates primarily in the membranes of low accumulators

Next, we set out to determine key phenotypic differences between low and high tachyplesin-NBD accumulators. Firstly, we found that *E. coli* low accumulators treated with 46 µg mL^{-1} tachyplesin-NBD for 60 min at 37 °C exhibited a fluorescence distribution akin to the entire population of cells treated with 46 µg mL^{-1} tachyplesin-NBD at 0 °C (Figure S4). At this low temperature, antimicrobials adhere non-specifically to bacterial surfaces as the passive and active transport across the bacterial membrane is significantly diminished [36]. Therefore, these data suggest that tachyplesin-NBD accumulates primarily on the outer membrane of low accumulators, whereas high accumulators exhibit AMP accumulation both on the membrane and intracellularly.

To test this hypothesis, we measured tachyplesin-NBD accumulation in individual *E. coli* cells using our microfluidics-based microscopy platform as detailed in previous studies [39–41]. Consistent with our flow cytometry data, we observed a bimodal accumulation of tachyplesin-NBD (Figure S5), with the presence of low and high accumulators (Figure 1G-I, blue stars and red diamonds, respectively). In line with our hypothesis above, low accumulators exhibited a significantly higher membrane to cell centre fluorescence ratio compared to high accumulators (p-value < 0.0001, Figure 1J and 1K).

We also compared the forward scatter and violet side scatter values and cell lengths between low and high tachyplesin-NBD accumulators. We found that these two phenotypes had similar forward and violet side scatter values (Figure S6A and S6B), and high accumulators were significantly smaller than low accumulators in the microfluidics platform (p-value < 0.0001, Figure S6C). These data clearly exclude the possibility that high tachyplesin-NBD fluorescence is attributed to larger cell sizes.

Taken together, these data suggest the intriguing hypothesis that tachyplesin accumulation on the bacterial membrane may not be sufficient for bacterial eradication.

Tachyplesin accumulation in the bacterial membrane is insufficient for bacterial eradication

To test the hypothesis that membrane accumulation of tachyplesin may be insufficient for bacterial eradication, we conducted simultaneous measurements of tachyplesin-NBD accumulation and its efficacy in bacterial eradication.

Stationary phase *E. coli* was treated with 46 µg mL^{-1} tachyplesin-NBD for 60 min followed by fluorescence-activated cell sorting to separate low and high accumulators, alongside untreated control cells (Figure 2A, 2B, S7 and Methods). Subsequently, we assessed the survival of each sorted sample via colony-forming unit assays and found that the survival fraction of low accumulators was not significantly different of that measured for untreated cells, whereas the survival fraction of high accumulators was significantly lower (p-value < 0.0001, Figure 2C).

To investigate the hypothesis that tachyplesin causes less membrane damage to low accumulators, we employed our microfluidics-based microscopy platform to simultaneously measure tachyplesin-NBD accumulation and bacterial membrane integrity via propidium iodide (PI) [42]. We found a strong positive semi-log correlation between tachyplesin-NBD fluorescence and PI fluorescence (r^2 = 0.70, p-value < 0.0001): all bacteria exhibiting PI staining had a tachyplesin-NBD fluorescence greater than 800 a.u. and were high

accumulators characterised by tachyplesin-NBD localisation both on the bacterial membrane and intracellularly; by contrast, low accumulators, where tachyplesin-NBD primarily localised on the bacterial membrane, did not stain with PI (Figure 2D-G).

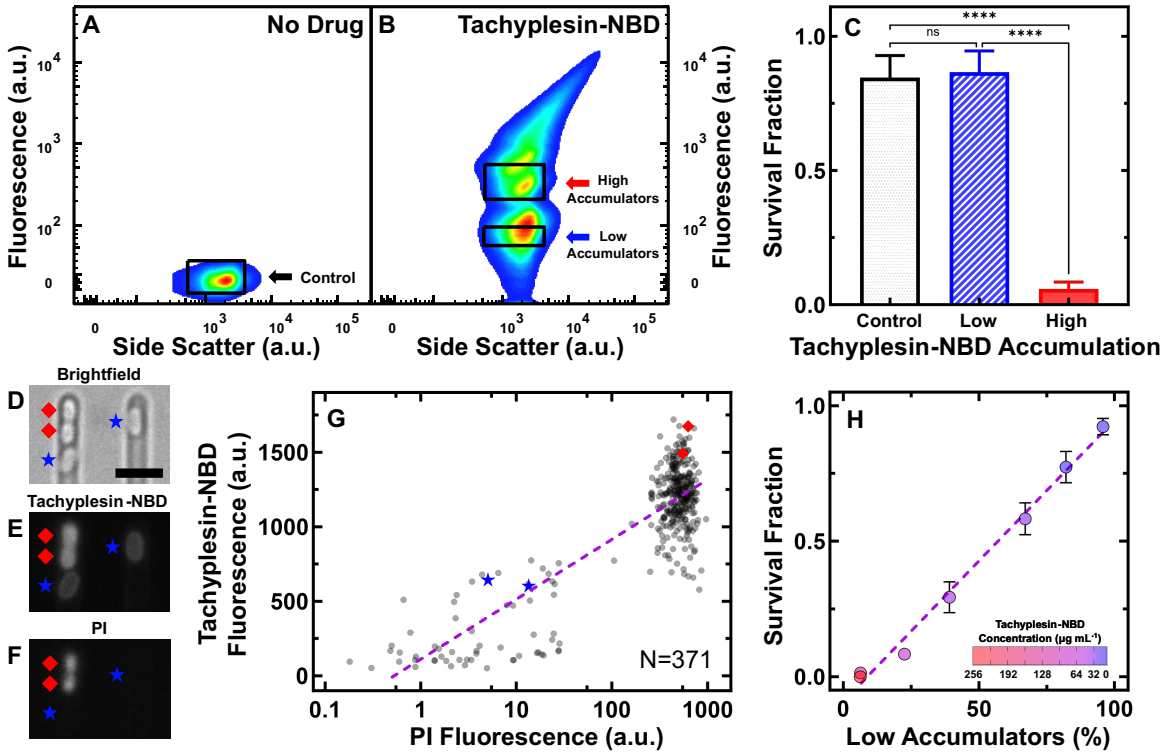

Figure 2. Intracellular tachyplesin accumulation is essential for its antimicrobial efficacy. (A and B) Fluorescence and side scatter values of individual *E. coli* treated with M9 at 37 °C for 60 min (A) and 46 µg mL^{-1} tachyplesin-NBD in M9 at 37 °C for 60 min (B). The black rectangles show the gates used to sort approximately one million untreated control cells, low and high tachyplesin-NBD accumulators for subsequent analysis. Data collected from four independent biological replicates. (C) Survival fraction of cells sorted through the untreated control, low and high tachyplesin-NBD accumulator gates presented in A and B. Bars and error bars represent the mean and standard deviation of data obtained from four biological replicates, each comprising three technical replicates. Statistical significance was assessed using an ordinary one-way ANOVA with Tukey's multiple comparisons test and confidence level at 95%. ****: $p<0.0001$, ns: $p>0.05$. (D-F) Representative microscopic images depicting brightfield (D), tachyplesin-NBD fluorescence (E), and propidium iodide (PI) fluorescence (F) after exposure to 46 µg mL^{-1} tachyplesin-NBD in M9 followed by a wash in M9 for 60 min, then 30 µM PI staining for 15 min at 37 °C in the microfluidic mother machine. Blue stars and red diamonds indicate low and high accumulators, respectively. Scale bar indicates 2.5 µm. (G) Correlation between tachyplesin-NBD and PI fluorescence of N = 371 individual *E. coli* cells collected from three independent biological replicates. The purple dashed line shows a nonlinear regression (semi-log) of the data ($r^2 = 0.70$, p-value < 0.0001). (H) Correlation between the proportion of low accumulators as a percentage of the whole bacterial population measured via flow cytometry and survival fraction measured via colony-forming unit assays. Symbols and error bars represent the mean and standard deviation of three independent biological replicates, each comprising three technical replicates. Tachyplesin-NBD treatment concentration indicated by colour gradient. Some error bars are masked by the data points. The purple dashed line illustrates a linear regression of the data ($r^2 = 0.98$, p-value < 0.0001).

Finally, we set out to determine the contribution of low accumulators to the overall population survival. We exposed stationary phase *E. coli* to increasing concentrations of tachyplesin-NBD for 60 min, while simultaneously measuring tachyplesin-NBD accumulation in individual bacteria and the bacterial eradication efficacy of tachyplesin-NBD at the population level. We used the accumulation data to measure the proportion of low accumulators relative to the whole population of bacteria at increasing tachyplesin-NBD concentrations and found a strong positive linear correlation with the population survival fraction (r^2 = 0.98, p-value < 0.0001, Figure 2H). Taken together, these data demonstrate that low accumulators primarily accumulate tachyplesin-NBD on the bacterial membrane, maintaining an intact membrane, strongly contributing to the survival of the bacterial population in response to tachyplesin treatment.

Enhanced efflux, membrane lipid alterations and increased secretion of outer membrane vesicles facilitate low accumulation of tachyplesin

Next, we set out to investigate the mechanisms underpinning low accumulation of tachyplesin. Following our established protocols [43,44], we performed genome-wide comparative transcriptome analysis between low accumulators, medium accumulators (containing a 1:1, *v/v* mixture of low and high accumulators), high accumulators, and untreated stationary phase bacteria that were sorted via fluorescence-activated cell sorting. Principal component analysis revealed clustering of the replicate transcriptomes of low, medium and high tachyplesin-NBD accumulators, and untreated control cell groups with a distinct separation between these groups on the PC1 plane (Figure S8).

Our analysis revealed significant differential expression of 1,623 genes in at least one of the three groups compared to untreated cells (Data Set S2). By performing cluster analysis of these genes [41,44] (see Methods), we identified five distinct clusters of genes based on their expression profile across the three groups relative to the control population (Figure 3A, 3B and Figure S9). Gene ontology enrichment revealed significant enrichment of biological processes in clusters 2, 4 and 5 (Figure 3C and 3D). Genes in cluster 2 and 5 were upregulated to a greater extent in high accumulators; genes in cluster 4 were downregulated to a greater extent in high accumulators. This analysis enabled the identification of major transcriptional differences between low and high accumulators of tachyplesin.

Firstly, low accumulators displayed a less translationally and metabolically active state. This is particularly evident in cluster 2 that includes processes involved in protein synthesis, energy production and gene expression that are upregulated to a greater extent in high accumulators than low accumulators (see Table S1 and Data Set S3 for a short and complete list of genes involved in these processes, respectively).

Secondly, low accumulators displayed enhanced membrane transport activity. This is particularly evident in cluster 4 that includes processes involved in transport of organic substances that are downregulated to a greater extent in high accumulators than low accumulators (Figure 3B and 3D). Notably, genes encoding major facilitator superfamily (MFS) and resistance-nodulation-division (RND) efflux pump components such as EmrK, MdtM and MdtEF, were upregulated in low accumulators (Table S1 and Data Set S3).

Thirdly, low and high accumulators displayed differential expression of biological pathways facilitating the synthesis and assembly of the lipopolysaccharides (LPS), which is the first site of interaction between gram-negative bacteria and AMPs [45]. For example, cardiolipin synthase B ClsB was contained in cluster 2 and downregulated in low accumulators (Table S1 and Data Set S3), suggesting that low accumulators might display a lower content of cardiolipin that

could bind tachyplesin since it is negatively charged. To further test this hypothesis, we performed a comparative lipidomic analysis of untreated versus tachyplesin-NBD treated stationary phase *E. coli* using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry [46]. In accordance with our transcriptomic data, we found that treatment with tachyplesin-NBD, caused a strong decrease of phosphatidylglycerol (PG from 37% to 3%, i.e. the main constituent of cardiolipin), along with the emergence of lysophosphatidylethanolamines (LPE, 10%) and slight increases in PE and FA (Figure 3E, Figure S10 and Table S2).

Fourthly, OmpA, OmpC, DegP, TolA, TolB and Pal were downregulated in low accumulators, and their deletion has previously been associated with increased secretion of outer membrane vesicles (OMVs) that can confer resistance to AMPs and small molecule antibiotics [47,48]. Moreover, NlpA, LysS, WaaCF and Hns were upregulated in low accumulators, and their overexpression has been previously associated with enhanced OMV secretion [49,50].

Finally, low accumulators displayed an upregulation of peptidases and proteases compared to high accumulators, suggesting a potential mechanism for degrading tachyplesin (Table S1 and Data Set S3).

Next, we sought to investigate transcription factor (TF) activities via differential expression of their known regulatory targets [51]. A total of 126 TFs were inferred to exhibit differential activity between low and high accumulators (Data Set S4). Among the top ten TFs displaying higher inferred activity in low accumulators compared to high accumulators, five regulate transport systems, i.e. Nac, EvgA, Cra, NtrC and MarA (Figure 3F). Most notably, EvgA, which serves as a response regulator in the EvgAS two-component regulatory system, regulates *mdtE*, *mdtF*, *emrK* and *yfdX*, encoding components of three distinct efflux pumps [52]. However, the deletion of *evgA* alone, or any of the 20 TFs with strong activity observed in either low or high accumulators (Figure 3F), did not lead to a distribution of tachyplesin-NBD accumulation differing from that of the *E. coli* parental strain (Figure S11), suggesting that an intricate interplay between multiple TFs might contribute to the formation of low accumulators.

Taken together these data suggest that phenotypic variants within clonal bacterial populations display a differential regulation of metabolic activity, lipid composition, efflux, outer membrane vesicle secretion and proteolytic processes in order to avoid tachyplesin accumulation.

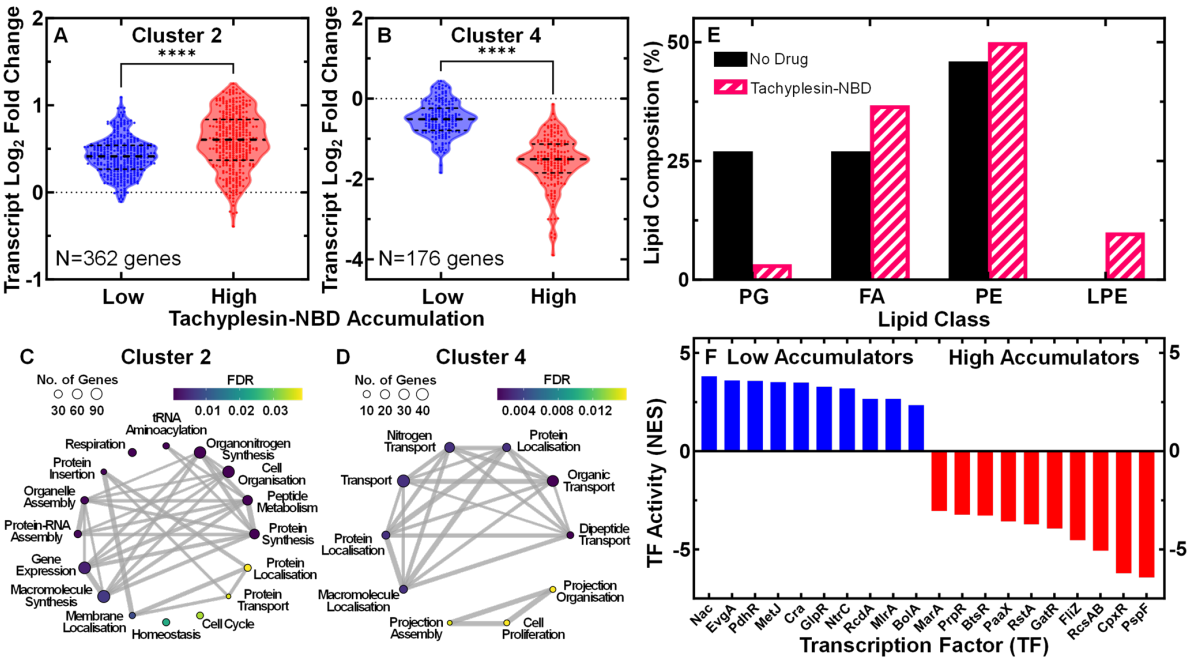

Figure 3. Biological processes facilitating low tachyplesin accumulation. (A and B) Log_2 fold changes in transcript reads of genes in low and high tachyplesin-NBD accumulators (blue and red violin plots, respectively) relative to untreated stationary phase *E. coli* bacteria in cluster 2 (A) and cluster 4 (B). Each dot represents a single gene, dashed lines indicate the median and quartiles of each distribution. Dotted lines represent a log_2 fold change of 0. Statistical significance was tested using a paired two-tailed Wilcoxon nonparametric test (due to non-normally distributed data) with a two-tailed p-value and confidence level at 95%. ****: p-value < 0.0001. The full list of genes belonging to each cluster are reported in Data Set S2 and the violin plots of log_2 fold changes for all clusters are shown in Figure S9. Data was collected from four independent biological replicates then pooled for analysis. (C and D) Corresponding gene ontology enrichment plots of biological processes enriched in cluster 2 and 4. Each dot represents a biological process with its size indicating the number of genes associated with each process and the colour indicating the false discovery rate (FDR). The lines and their thickness represent the abundance of mutual genes present within the connected biological processes. The enrichment plot for cluster 5 is not shown as only one biological process, "response to biotic stimulus", was enriched. Full details about each process are reported in Data Set S3. (E) Relative abundance of lipid classes (PG, phosphatidylglycerol; FA, fatty acids; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine) in untreated bacteria and bacteria treated with 46 µg mL^{-1} tachyplesin-NBD for 60 min. (F) Inferred transcription factors (TFs) activity, reported as normalised enrichment scores (NES), for the ten TFs with highest inferred activity in low or high accumulators (blue and red bars, respectively). Activity was inferred using Data Set S2 and a full list of TFs and the genes they regulate within each cluster is reported in Data Set S4.

Low accumulators respond to tachyplesin treatment by enhancing efflux

Next, we set out to delve deeper into the specific molecular mechanisms utilised by low accumulators in response to tachyplesin exposure. We hypothesised that should modifications to lipid composition or enhanced secretion of OMVs be responsible for low tachyplesin accumulation, we should observe consistently low tachyplesin-NBD fluorescence in the intracellular space of low accumulators. Conversely, should efflux or proteolytic activities by proteases underpin the functioning of low accumulators, we should observe high initial tachyplesin-NBD fluorescence in the intracellular space of low accumulators followed by a

decrease in fluorescence due to efflux or proteolytic degradation. To test this hypothesis, we investigated the accumulation kinetics of tachyplesin-NBD in individual stationary phase *E. coli* cells after different durations of tachyplesin-NBD treatment. Strikingly, within just 15 min of treatment, we observed high levels of tachyplesin-NBD accumulation in all cells, placing the whole population in the high accumulator group with a median fluorescence of 15,000 a.u. (Figure 4A). However, after 30 min of treatment, a subpopulation began to display lower fluorescence, and after 60 min, the low accumulator fluorescence distribution became evident with a median fluorescence of 3,600 a.u. (Figure 4A). By contrast, the median fluorescence of high accumulators increased from 15,000 a.u. at 15 min to 32,000 a.u. after 120 min (Figure 4A). Taken together, these data demonstrate that the entire isogenic *E. coli* population initially accumulates tachyplesin-NBD to high levels, but a subpopulation can reduce intracellular accumulation of the drug while the other subpopulation continues to accumulate the drug to higher levels. Therefore, these data support the hypothesis that either enhanced efflux or proteolytic activity is employed by low accumulators as a primary response to tachyplesin exposure. Furthermore, it is conceivable that such responses are induced by tachyplesin exposure since low accumulators form following initial high tachyplesin accumulation in all cells.

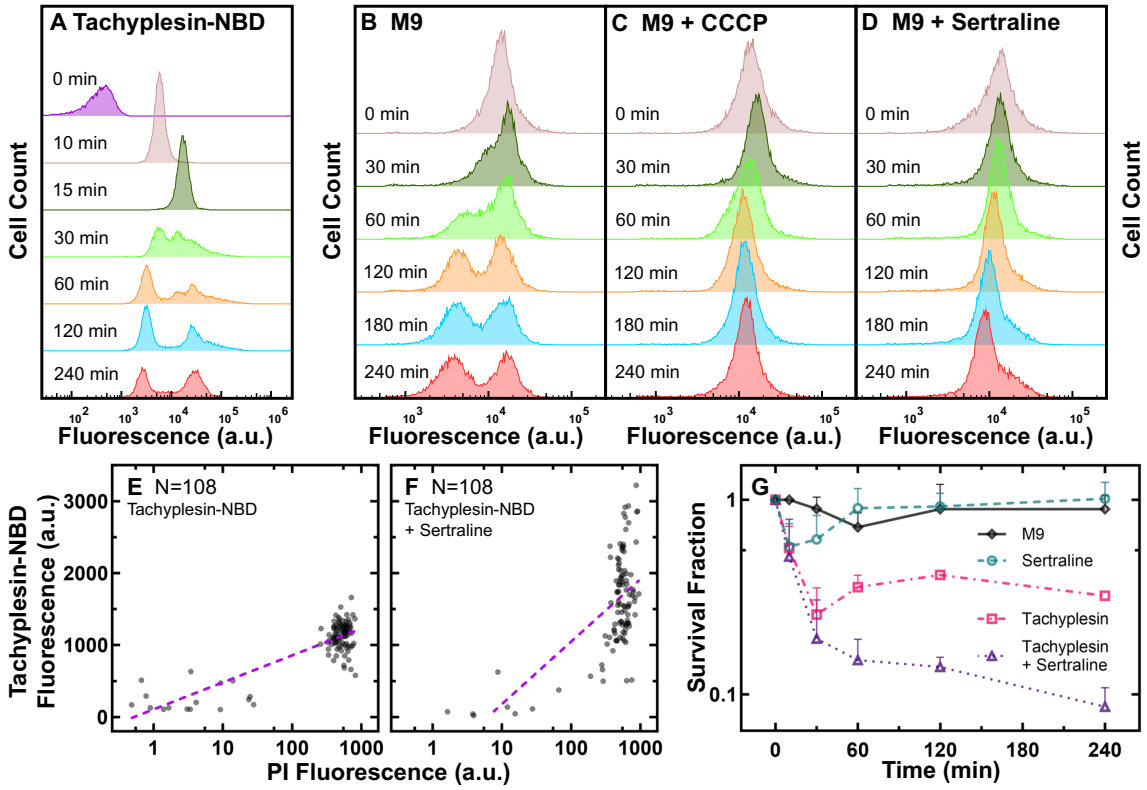

Figure 4. Low accumulators respond to tachyplesin treatment by enhancing efflux. (A) Distribution of tachyplesin-NBD accumulation in stationary phase *E. coli* over 240 min of treatment with 46 µg mL^{-1} tachyplesin-NBD in M9 at 37 °C. (B-D) Distribution of tachyplesin-NBD accumulation over 240 min after 15 min pretreatment in 46 µg mL^{-1} tachyplesin-NBD in M9 then washed and transferred into M9 (B), CCCP (50 µg mL^{-1}) (C), or sertraline (30 µg mL^{-1}) (D). Graphs are representative of three independent biological replicates and report 10,000 events. (E and F) Correlation of tachyplesin-NBD and PI fluorescence of individual stationary phase *E. coli* cells measured in the microfluidic mother machine after 60 min of 46 µg mL^{-1} tachyplesin-NBD treatment (N=108) (E) or cotreatment with 46 µg mL^{-1} tachyplesin-NBD and 30 µg mL^{-1} sertraline (N=108) (F). Purple dashed lines show nonlinear (semi-log) regressions (r^2 = 0.68 and 0.38, respectively). (G) Survival fraction of stationary phase *E. coli* over 240 min treatment with tachyplesin (46 µg mL^{-1}, magenta squares) or sertraline (30 µg mL^{-1}, green circles), cotreatment with tachyplesin and sertraline (46 µg mL^{-1} and 30 µg mL^{-1}, respectively, purple triangles), or incubation in M9 (black diamonds). Symbols and error bars indicate the mean and standard deviation of measurements performed in three biological replicates consisting of three technical replicates each. Some error bars are masked behind the symbols.

Tachyplesin accumulation and efficacy can be boosted with the addition of efflux pump inhibitors or nutrients

To further investigate the role of efflux in low tachyplesin accumulation, we first incubated stationary phase *E. coli* in tachyplesin-NBD and then resuspended them either in minimal medium M9 or M9 containing 50 µg mL^{-1} carbonyl cyanide m-chlorophenyl hydrazone (CCCP), an ionophore that disrupts the proton motive force and is commonly employed to abolish antimicrobial efflux. We observed the emergence of the low accumulator distribution only in the absence but not in the presence of CCCP (Figure 4B and 4C, respectively), further demonstrating that low accumulators employ enhanced efflux as a primary response to exposure to tachyplesin. However, it is also conceivable that besides enhanced efflux, low accumulators employ proteolytic activity, OMV secretion and variations to their LPS as secondary responses to hinder further uptake and intracellular accumulation of tachyplesin. Since CCCP is cytotoxic to mammalian cells and has been limited to laboratory use only [53], we screened a panel of alternative efflux pump inhibitors (EPIs) to identify compounds capable of preventing the formation of low accumulators of tachyplesin-NBD. Interestingly, M9 containing 30 µg ml^{-1} sertraline, an antidepressant which inhibits efflux activity of RND pumps [54] was able to prevent the emergence of low accumulators (Figure 4D and Figure S12). Furthermore, sertraline cotreatment with tachyplesin-NBD simultaneously increased tachyplesin-NBD accumulation and PI fluorescence levels in individual cells (Figure 4E and 4F, p-value < 0.0001 and 0.05, respectively).

Next, we investigated whether sertraline could enhance the bactericidal activity of the parent drug, tachyplesin. Using colony-forming unit assays, we measured the survival fraction of stationary phase *E. coli* treated with either tachyplesin at 46 µg mL^{-1} or sertraline at 30 µg mL^{-1}, or a combination of both compounds, and compared these data to untreated *E. coli* incubated in M9. The survival fraction after treatment with sertraline was comparable to that measured for untreated *E. coli*, whereas the survival fraction measured after the combination treatment was 5-fold lower than that measured after tachyplesin monotherapy (Figure 4G).

Our transcriptomics analysis also suggested that low tachyplesin accumulators are less metabolically active compared to high accumulators. Therefore, we set out to test the hypothesis that the formation of low accumulators could be prevented by manipulating the environment around the bacteria. We treated stationary phase *E. coli* in 46 µg mL^{-1} tachyplesin-NBD either in M9, or M9 supplemented with 0.4% glucose and 0.2% casamino acids. After 15 min of treatment, both conditions resulted in a unimodal fluorescence distribution, with median values of 15,000 a.u. and 18,000 a.u., respectively (Figure S13 and S13B). However, upon extending the treatment to 30 min, we observed distinct responses to tachyplesin-NBD treatment. For *E. coli* treated with tachyplesin-NBD in M9, the emergence of low accumulators led to a bimodal distribution of single-cell fluorescence. In contrast, when *E. coli* was treated with tachyplesin-NBD in M9 supplemented with glucose and casamino acids, the fluorescence of the entire population increased, resulting in a unimodal distribution of single-cell fluorescence with a median of 35,000 a.u. (Figure S13B).

Similarly, *E. coli* incubated in lysogeny broth (LB) for 3 h before drug treatment, and therefore in the exponential phase of growth, displayed a unimodal fluorescence distribution with a median of 80,000 a.u. after 30 min treatment in tachyplesin-NBD in M9 (Figure S12C). These exponential phase bacteria also displayed a modest increase in cell size compared to stationary phase *E. coli* (Figure S14). Interestingly, a small subpopulation of low accumulators emerged within exponential phase *E. coli* after 120 min tachyplesin-NBD treatment M9, with a median of 4,700 a.u. (Figure S13C). This subpopulation contributed to 10% of the entire isogenic *E. coli* subpopulation, five-fold less abundant compared to stationary phase *E. coli*

treated in tachyple7sin-NBD in M9 (Figure S13A). Finally, we investigated whether preventing the formation of low accumulators via nutrient supplementation could enhance the efficacy of tachyplesin treatment by performing colony-forming unit assays. We observed that tachyplesin exhibited enhanced efficacy in eradicating *E. coli* both when the medium was supplemented with glucose and casamino acids, and against exponential phase *E. coli* compared to stationary phase *E. coli* without nutrient supplementation (Figure S15).

Discussion

The link between antimicrobial accumulation and efficacy has not been widely investigated, especially in the context of AMPs [25]. In fact, it has been widely accepted that the cell membrane is the primary target of most AMPs and that their main mechanism of action is membrane disruption. However, emerging evidence suggests that AMPs may also enter the intracellular environment and target intracellular processes [5,55,56].

Here, we demonstrate for the first time that there is a strong correlation between tachyplesin intracellular accumulation and efficacy and that tachyplesin accumulates intracellularly only in bacteria that do not survive tachyplesin exposure. These findings align with evidence showing that tachyplesin interacts with intracellular targets such as the minor groove of DNA [24,57], intracellular esterases [58], or the 3-ketoacyl carrier protein reductase FabG [23]. However, our transcriptomic analysis did not reveal differential regulation of any of these pathways between low and high accumulators, suggesting that bacteria likely employ alternative mechanisms rather than target downregulation to survive tachyplesin treatment, a point on which we expand below.

The emergence of genetic resistance to AMPs is strikingly lower compared to resistance to small molecule antibiotics [7,8,59]. However, research on AMP resistance has often focused on whole population responses [7,8,59,60], overlooking heterogeneous responses to drug treatment in isogenic bacteria. Crucially, phenotypic variants can survive treatment with small molecule antibiotics through several distinct phenomena, including persistence [61], the viable but nonculturable state [62], heteroresistance [63], tolerance [64] and perseverance [17].

Here, we report the first observation of phenotypic resistance to tachyplesin within isogenic *E. coli* and *P. aeruginosa* populations. This novel phenomenon is characterised by a bimodal distribution of tachyplesin accumulation, revealing two distinct subpopulations: low (surviving) and high (susceptible) accumulators. This bimodal distribution draws similarities to the perseverance phenomenon identified for two antimicrobials possessing intracellular targets, rifampicin and nitrofurantoin. In fact, perseverance is characterised by a bimodal distribution of single-cell growth rates, where the slower-growing subpopulation maintains growth during drug treatment [17]. Our data suggests that such slower-growing subpopulations might display lower antibiotic accumulation and thus enchanced survival to antibiotic treatment.

Indeed, bacteria can reduce the intracellular concentration of antimicrobials via active efflux, allowing them to survive in the presence of high extracellular antibiotic concentrations [65]. Efflux as a mechanism of tachyplesin resistance has been shown at the population level in tachyplesin-resistant *P. aeruginosa* via transcriptome analysis [60,66], whereas phenotypic resistance to tachyplesin has not been investigated. Heterogeneous expression of efflux pumps within isogenic bacterial populations has been reported [29,32,33,67–69]. However, recent reports have suggested that efflux is not the primary mechanism of antimicrobial resistance within stationary phase bacteria [31,70] and the impact of heterogeneous efflux pump expression on AMP accumulation is unknown. Here, we show for the first time the upregulation of a range

of MFS, ABC and RND efflux pumps in a stationary phase subpopulation that exhibited low accumulation of tachyplesin with corresponding enhanced survival.

Moreover, whether certain resistance mechanisms utilised by phenotypic variants surviving antimicrobial treatment are preemptive or induced by drug treatment is underinvestigated [61,71] primarily due to the requirement of prior selection for phenotypic variants [29]. As our accumulation assay did not require the prior selection for phenotypic variants, we have unambiguously demonstrated that low accumulators emerge subsequent to the initial high accumulation of tachyplesin-NBD, indicating enhanced efflux as an induced response.

Furthermore, most AMPs are cationic and interact electrostatically with the negatively charged LPS in the bacterial outer membrane [72]. To reduce affinity to AMPs, bacteria can modify their membrane charge, thickness, fluidity, or curvature [73]. At the population level, bacteria have been reported to add positively charged L-Ara4N or phosphoethanolamine (pEtN) to LPS lipid-A to resist AMPs [59]. Accordingly, our transcriptomics analysis revealed the upregulation in the low accumulator subpopulation of the *arn* operon that is involved in the biosynthesis and attachment of the negatively charged L-Ara4N to lipid-A [74]. Furthermore, tachyplesin has been shown to bind strongly to negatively charged PG lipids [75]. Accordingly, our lipidomics data revealed that tachyplesin-treated bacteria were composed of significantly lower abundance of PG lipids compared to the untreated bacteria.

OMVs, nano-sized proteoliposomes secreted by Gram-negative bacteria, are a less studied system that allows bacteria to remove AMPs from the cell [76]. Increased secretion of OMVs has also been shown to be induced by AMP exposure [48]. OMVs are suggested to counteract AMPs via at least three mechanisms: the adsorption of extracellular AMPs to decrease AMP concentrations in the environment [47,48]; removal of parts of the bacterial outer membrane affected by AMPs [48]; and export of antimicrobials from the intracellular compartment [77]. Our transcriptomic data revealed the downregulation in low accumulators of genes, such as *ompAC*, known to increase the secretion of OMVs upon deletion and the upregulation of genes, such as *nlpA*, whose overexpression has been linked to enhanced OMV secretion [78].

Bacteria also employ extracellular or intracellular proteases [6,59] and peptidases [79] to cleave and neutralise the activity of AMPs including tachyplesin [80]. Our transcriptomic analysis revealed the simultaneous upregulation in low accumulators of proteases and peptidases that could potentially degrade tachyplesin either extracellularly or intracellularly.

Responses to extrinsic environmental cues, such as the starvation-induced stringent response, have been shown to induce failure in antimicrobial treatment [81]. These responses are typically mediated by two-component regulatory systems (TCSs) sensing external stimuli via sensor kinases and altering gene expression via response regulators [82]. Additionally, nucleoid associated proteins (NAPs) influence bacterial chromosome organisation and gene transcription in response to intra- or extracellular stress factors [83]. Finally, transcriptional regulators such as regulators of the LysR family and histone-like proteins (H-NS) also modulate gene expression [84].

We discovered that low accumulators display a greater activity of EvgA a transcription factor that serves as the response regulator of the EvgAS TCS, which upregulates resistance genes, including multidrug efflux pumps EmrKY, MdtEF, AcrAB and MdfA [85]. Furthermore, several other TCSs and NAPs that regulatie resistance mechanisms relevant to efflux, membrane modifications, OMV secretion, protease and peptidase displayed a greater activity in low accumulators. Drug accumulation assays performed with single-deletion mutants of the TFs above did not prevent the formation of low accumulators, suggesting that their functioning may

be simultaneously controlled by multiple TFs [86], or that gene duplications or compensatory mechanisms are present in the Keio collection [87].

Drugs that inhibit the efflux activity of bacterial pathogens have been shown to be a promising strategy for repurposing antimicrobials [88–90]. An FDA-approved drug, sertraline, was found to be effective in preventing the formation of the low accumulator phenotype by inhibiting tachyplesin efflux and enhancing the eradication of stationary phase bacteria. These data strongly suggest that EPIs are a promising approach for developing new combination therapies against stationary phase bacteria, contrary to previous evidence suggesting their ineffectiveness against stationary phase bacteria [31]. However, it should be noted that the concentration of sertraline in the plasma of patients using sertraline as an antidepressant is below the concentration that we employed in our study [91]. Therefore, more investigation should be carried out to further optimise the use of sertraline or other EPIs in combination with tachyplesin.

Moreover, growth rate and metabolism have been shown to influence antimicrobial efficacy [92], with starved or slow growing stationary phase bacteria becoming transiently insensitive to antimicrobials when metabolites or ATP become unavailable [93]. Due to the heightened resistance of starved and stationary phase cells, successful attempts have been made to selectively target metabolically dormant bacteria [94] or to sensitise recalcitrant cells via metabolites as a potential therapeutic strategy [95]. Likewise, we found that stationary phase cells supplemented with glucose and casamino acids were more sensitive to treatment with tachyplesin. However, nutrient supplementation may potentially lead to an increase in bacterial cell count and disease burden, as well as an escalation in selection pressure on surviving cells [3]. Therefore, we conclude that the use of EPIs in combination with tachyplesin represents a more viable antimicrobial treatment against antimicrobial-refractory stationary phase bacteria.

METHODS

Chemicals and cell culture

All chemicals were purchased from Fisher Scientific or Sigma-Aldrich unless otherwise stated. LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl) and LB agar plates (LB with 15 g L^{-1} agar) were used for planktonic growth and streak plates. Carbon-free M9-minimal medium used for dilution of antimicrobials, EPIs and bacteria was prepared using 5×M9 minimal salts (Merck, Germany), with an additional 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$ in Milli-Q water. 0.4% glucose and 0.2% casamino acids were added to yield nutrient-supplemented M9. M9 was then filtered through a 0.22 µm Minisart® Syringe Filter (Sartorius, Germany). Tachyplesin-1 and tachyplesin-1-NBD were synthesised by WuXi AppTech (Shanghai, China). Stock solutions of antimicrobials were obtained by dissolving in Milli-Q water at a concentration of 1.28 mg mL^{-1} and EPIs were obtained by dissolving in dimethyl sulfoxide at a concentration of 1 mg mL^{-1}.

E. coli BW25113 and *S. aureus* ATCC 25923 were purchased from Dharmacon™ (Horizon Discovery, UK). *E. coli* clinical isolates and *K. pneumoniae* (JS1187) were kindly provided by Fernanda Paganelli, UMC Utrecht, the Netherlands. *P. aeruginosa* (PA14 *flgK*::Tn5B30(Tc)) was kindly provided by George O'Toole, Dartmouth College, USA. *E. coli* KO mutants were obtained from the Keio collection [96]. All strains were stored in a 50% glycerol stock at -80 °C. Streak plates for each strain were produced by thawing a small aliquot of the corresponding glycerol stock every week and plated onto LB agar plates. Stationary phase cultures were prepared by inoculating 100 mL fresh LB medium with a single bacterial colony from a streak plate and incubated on a shaking platform at 200 rpm and 37 °C for 17 h.

Exponential phase cultures were prepared by transferring 100 µL of a stationary phase culture into 100 mL LB and incubated on a shaking platform at 200 rpm and 37 °C for 3 h.

Synthesis of fluorescent antimicrobial derivatives

Fluorescent antimicrobial derivatives of tachyplesin [34,38], arenicin [97], polymyxin-B [98] and octapeptin [99] were designed and synthesised based on structure-activity-relationship studies and synthetic protocols reported in previous publications, substituting a non-critical amino acid residue with an azidolysine residue that was then employed for the subsequent Cu-catalysed azide-alkyne cycloaddition 'click' reactions with nitrobenzoxadiazole (NBD)-alkyne. Detailed synthesis and characterisation of these probes will be reported in due course.

Phylogenetic and AMR genes analyses

The *E. coli* BW25113 genome sequence was downloaded from the National Center for Biotechnology Information's (NCBI) GenBank® [100]. *E. coli* clinical isolates MA02514, MA07534, MA08141, MC04960 and MC42862 were collected and sequenced by UMC Utrecht, the Netherlands [101]. Briefly, genomic DNA libraries were prepared using the Nextera XT Library Prep Kit (Illumina, USA) and sequenced on either the Illumina MiSeq or NextSeq (Illumina, USA). Contigs were assembled using SPAdes Genome Assembler version 3.6.2 [102], and contigs larger than 500 bp with at least 10× coverage were further analysed. *E. coli* clinical isolates JS1060, JS1073, JS1147 and JS1147 were collected by UMC Utrecht, the Netherlands, and were sequenced by a microbial genome sequencing service (MicrobesNG, UK). Briefly, genomic DNA libraries were prepared using the Nextera XT Library Prep Kit (Illumina, USA) following the manufacturer's protocol with the following modifications: input DNA was increased two-fold, and PCR elongation time is increased to 45 s. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG, Switzerland). Libraries were sequenced on an Illumina NovaSeq 6000 (Illumina, USA) using a 250 bp paired end protocol. Reads and adapter trimmed using Trimmomatic version 0.30 [103] with a sliding window quality cutoff of Q15. *De novo* assembly was performed on samples using SPAdes version 3.7 [102], and contigs were annotated using Prokka 1.11 [104].

Genomic data for AMR genes were screened through ABRicate [105] and the NCBI's AMRFinderPlus databases [106]. A rooted phylogenetic tree was created from the genomic data by pairwise distance estimation of all genomes and an outgroup genome (NCBI's reference genome for *Escherichia fergusonii*) with the *dist* function from the Mash package [107], with a k-mer size of 21. Unrooted phylogenetic trees were created from the resulting pairwise distance matrices using the *nj* (neighbour joining) function in APE [108]. These trees were then rooted to the outgroup (*ape::root*). The phylogenetic tree was plotted against presence/absence heatmaps of AMR genes using *ggtree* [109].

Flow cytometric accumulation and efflux assays

Bacterial cultures were prepared as described above and transferred to a microcentrifuge tube for centrifugation in a SLS4600 microcentrifuge (Scientific Laboratory Supplies, UK) at 4,000 ×*g* and room temperature for 5 min. For accumulation assays, bacterial cultures were adjusted to an OD_{600} of 4 for *E. coli* and *K. pneumoniae*, 5 for *S. aureus*, and 2 for *P. aeruginosa* by removing the supernatant and resuspending in the fluorescent antimicrobial derivative diluted with carbon-free or nutrient-supplemented M9 at a final volume between 50 and 550 µL depending on the experiment. Treatments were incubated at 37 °C and 1,000 rpm on a ThermoMixer® C (Eppendorf, Germany) and on ice for cold treatments. 30 µL sacrificial aliquots were then transferred to microcentrifuge tubes at appropriate time points. A wash step was immediately performed on the sacrificial aliquots to eliminate any unaccumulated drug via centrifugation at 4000 ×*g* for 5 min and its supernatant removed. For efflux assays, *E. coli* was incubated in 46 µg mL^{-1} tachyplesin-NBD for 15 min before carrying out a wash step and incubation in EPI diluted in carbon-free M9. Carbon-free M9 was used for untreated control treatments in both accumulation and efflux assays. Cells were then resuspended in carbon-free M9 and diluted 500× before flow cytometry measurements. For the accumulation assays, to account for time between taking sacrificial aliquots and flow cytometric measurement, 10 min was added to the reported incubation time in figures for the first two time points (i.e. the sacrificial aliquot taken immediately after tachyplesin-NBD addition was reported as 10 min).

Flow cytometric measurements were performed on the CytoFLEX S Flow Cytometer (Beckman Coulter, USA) equipped with a 488 nm (50 mW) and a 405 nm (80 mW) laser. Fluorescence of individual bacteria was measured using the fluorescein isothiocyanate (FITC-A) channel (488 nm 525/40BP) and cell size was measured using FSC (forward scatter) and Violet SSC (side scatter) channels. Avalanche photodiode gains of FSC: 1,000, SSC: 500, Violet SSC: 1, FITC: 250 and a threshold value of SSC-A: 10,000 to limit background noise was used. Bacteria were gated to separate cells from background noise by plotting FSC-A and Violet SSC-A (Figure S1A). Background noise was then further separated based on cellular autofluorescence measured on the FITC-A channel for cells not treated with fluorescent-peptides (Figure S1B). An additional gate on the FITC-A channel was then used for cells treated with fluorescent-peptides to further separate background noise (Figure S1C). CytoFLEX Sheath Fluid (Beckman Coulter, USA) was used as sheath fluid. Data was collected using CytExpert software (Beckman Coulter, USA) and exported to FlowJo™ version 10.9 software (BD Biosciences, USA) for analysis.

Microfluidic mother machine device fabrication

Microfluidic mother machine devices were fabricated by pouring a 10:1 (base:curing agent) polydimethylsiloxane (PDMS) mixture [110] into an epoxy mould kindly provided by S. Jun [111]. Each mother machine device contains approximately 6,000 bacterial hosting channels with a width, height, and length of 1, 1.5 and 25 µm, respectively. These channels are connected to a main microfluidic chamber with a width and height of 25 and 100 µm, respectively. After degassing the PDMS mixture in a vacuum desiccator (SP Bel-Art, USA) with a two-stage rotary vane pump FRVP series (Fisher Scientific, USA), the PDMS was cured at 70 °C for 2 h and peeled from the epoxy mould to obtain 12 individual chips [112]. Fluidic inlet and outlet were achieved using a 0.75 mm biopsy punch (WellTech Labs, Taiwan) at the two ends of the main chamber of the mother machine. The PDMS chip was then washed with ethanol and dried with nitrogen gas, followed by removal of small particles using adhesive tape (3M, USA) along with a rectangular glass coverslip (Fisher Scientific, USA). The microfluidic device was assembled by exposing the PDMS chip and glass coverslip to air plasma treatment at 30 W plasma power for 10 s with the Zepto One plasma cleaner (Diener Electronic, Germany) and then placed in contact to irreversibly bind the PDMS chip and glass coverslip [113]. The mother machine was then filled with 5 µL of 50 mg mL $^{-1}$ bovine serum albumin and incubated at 37 °C for 1 h to passivate the charge within the channels after plasma treatment, screening electrostatic interaction between bacterial membranes, PDMS and glass surfaces [114].

Microfluidics-microscopy assay to measure tachyplesin-NBD accumulation and PI staining in individual *E. coli*

A stationary phase *E. coli* culture was prepared as described above. The culture was adjusted to OD_{600} 75 by centrifuging 50 mL of culture in a conical tube at 3,220 ×*g* at room temperature for 5 min in a 5810 R centrifuge (Eppendorf, Germany). The supernatant was removed, and pellet resuspended in carbon-free M9 and vortexed. 5 µL of bacterial suspension was then injected into the mother machine device using a pipette and incubated at 37 °C for 30 to 60 min to allow for filling of ~80% of bacteria hosting channels [115] . The loaded microfluidic device was then mounted on an Olympus IX73 inverted microscope (Olympus, Japan) connected to a 60×, 1.2 N.A. objective UPLSAPO60XW/1.20 (Olympus, Japan) and a Zyla 4.2 sCMOS camera (Andor, UK) for visual inspection. Each region of interest was adjusted to visualise 23 bacteria hosting channels and a mean of 11 regions of interest were selected for imaging for each experiment via automated stages M-545.USC and P-545.3C7 (Physik Instrumente, Germany) for coarse and fine movements, respectively. Fluorinated ethylene propylene tubing (1/32" × 0.0008") was then inserted into both inlet and outlet accesses as previously reported [116]. The inlet tubing was connected to a Flow Unit S flow rate sensor (Fluigent, France) and MFCS-4C pressure control system (Fluigent, France) controlled via MAESFLO 3.3.1 software (Fluigent, France) allowing for computerised, accurate regulation of fluid flow into the microfluidic device. A 300 µL h^{-1} flow of carbon-free M9 for 8 min was used to clear the main channel of excess bacteria that had not entered the bacterial hosting channels. Tachyplesin-NBD (46 µg mL^{-1}) or tachyplesin-NBD (46 µg mL^{-1}) with sertraline (30 µg mL^{-1}) in carbon-free M9 was then flowed through the microfluidic device at 300 µL h^{-1} for 8 min, before reducing to 100 µL h^{-1} until 240 min. Carbon-free M9 was then flushed through the

device at 300 µL h^{-1} for 60 min to wash away unaccumulated tachyplesin-NBD. PI staining was performed by flowing PI (1.5 mM) at 300 µL h^{-1} for 15 min. Brightfield and fluorescence images were captured consecutively every 10 min until the end of the experiment by switching between brightfield and fluorescence mode in a custom LabVIEW software (National Instruments, USA) module. Fluorescence images were captured by illuminating bacteria with a pE-300^{white} broad-spectrum LED (CoolLED, UK) for 0.06 s on the blue excitation band at 20% intensity with a FITC filter for tachyplesin-NBD and green excitation band at 100 % intensity with a DAPI/TEXAS filter for PI. The entire experiment was performed at 37 °C in an environmental chamber (Solent Scientific, UK) enclosing the microscope and microfluidics equipment. A step-by-step guide to the process above can be found in [117].

Image and data analyses

Images were processed using ImageJ software as previously described [118]. Each individual bacterium was tracked throughout the 240 min tachyplesin-NBD treatment, 60 min carbon-free M9 wash and 15 min PI staining. To measure tachyplesin-NBD and PI accumulation, a rectangle was drawn around each bacterium in each brightfield image at every time point, obtaining its length, width, and relative position in the microfluidic hosting channel. The same rectangle was then overlaid onto the corresponding fluorescence image to measure the mean fluorescence intensity, which represents the total fluorescence normalised by cell size (i.e., the area covered by each bacterium in the 2D images), thus accounting for variations in tachyplesin-NBD and PI accumulation due to the cell cycle [119]. The same rectangle was then shifted to the nearest adjacent channel that did not contain any bacteria to measure the mean background fluorescence from extracellular tachyplesin-NBD in the media. This mean background fluorescence value was subsequently subtracted from the bacterium's mean fluorescence value. To measure tachyplesin-NBD fluorescence profiles, the fluorescence values of a straight horizontal line spanning 20 pixels (left to right) were recorded then converted and presented in µm. The membrane over cell centre fluorescence ratios were calculated by dividing the fluorescence of the membrane by the fluorescence value of the centre of the cell. The mean membrane fluorescence was obtained using the fluorescence of the seventh and thirteenth pixel, and the cell centre fluorescence using the tenth pixel along the profile measurements. To measure cell sizes of low and high accumulators, the vertical length of the rectangles drawn to measure fluorescence was taken for each cell then converted and presented in µm. All data were then analysed and plotted in Prism version 10.2.0 (GraphPad Software, USA).

Statistical significance tests, including the ordinary one-way ANOVA with Tukey's multiple comparisons tests, paired two-tailed Wilcoxon nonparametric test, unpaired two-tailed nonparametric Mann-Whitney U test, as well as linear and nonlinear (semi-log) regressions were calculated using Prism version 10.2.0 (GraphPad Software, USA).

Fluorescence-activated cell sorting

Samples for cell sorting were generated following the same protocol as flow cytometry accumulation assays. Cells were incubated for 60 min and a wash step carried out before performing a 30× dilution on 500 µL of treated cells for cell sorting. Cell sorting was performed on a BD FACSAria™ Fusion Flow Cytometer, equipped with a 488 nm (50 mW) laser (BD Biosciences, USA). Drops were generated at 70 psi sheath pressure, 87.0 kHz frequency, and 5.6 amplitude using a 70 µm nozzle. BD FACSFlow™ (BD Biosciences, USA) was used as sheath fluid and the instrument was set up for sorting with the BD™ Cytometer Setup & Tracking Beads Kit, and BD FACS™ Accudrop Beads reagents (BD Biosciences, USA), following the manufacturer's instructions. Cells were sorted based on their fluorescence measured on the 488 nm 530/30BP channel with PMT voltages of FSC: 452, SSC: 302 and 488 nm 530/30BP: 444 and a threshold value of 200 was applied on the SSC channel to limit background noise. Cell aggregates were excluded by plotting SSC-W against SSC-H (Figure S5A). Tachyplesin-NBD treated cells were sorted into two groups (low and high accumulators) and the untreated control treatment through a control gate (Figure S5E and S5C, respectively). A one-drop sorting envelope with purity rules was used. Approximately 1,000,000 million cells for each group were sorted directly into RNAprotect® (Qiagen, Germany) to stabilise RNA in preparation for extraction and into carbon-free M9 for lipid Folch extractions. An additional combined sample containing a 1:1, *v/v* mixture of sorted low and high accumulators was generated (referred to as medium accumulators) to aid and validate downstream transcriptomics analyses. Cells were sorted into carbon-free M9 for cell

viability measurements following the same protocol above. Data was collected via BD FACSDiva™ version 8.0.1 (BD Biosciences, USA) software and analysed using FlowJo™ version 10.9 software (BD Biosciences, USA). Post-sort analyses revealed high levels of purity and very low occurrences of events measured outside of the sorting gate for sorted cells (Figure S5D and S5F-H). Increased events measured in the M9 gate in post-sort samples are due to a reduced concentration of cells in post-sort analyses.

Cell viability assays

Cell viability was assessed by diluting cells in carbon-free M9, then plating on LB agar plates. Plates were then incubated at 37 °C for 17 h followed by colony forming unit counts. For flow cytometry accumulation and time-kill assays, sacrificial 30 µL aliquots of cells were obtained from the treatment microcentrifuge tube at their respective time points for dilution and spread plating. For cells sorted via fluorescence-activated cell sorting, low and high tachyplesin-NBD accumulators, and untreated control cells were sorted into carbon-free M9 before dilution and spread plating.

RNA extraction and sequencing

RNA extractions were performed on cells sorted into RNAprotect® (Qiagen, Germany). Enzymatic lysis and proteinase K digestion of bacteria was performed following protocol 4 in the RNAprotect® Bacteria Reagent Handbook (Qiagen, Germany). Purification of total RNA from bacterial lysate using the RNeasy® Mini Kit (Qiagen, Germany) following protocol 7 in the handbook. Due to the small quantity of initial material from cell sorting, RNeasy MinElute® spin columns were used instead of RNeasy® Mini spin columns. A further on-column DNase digestion using the RNase-Free DNase set (Qiagen, Germany) was performed following appendix B in the handbook. RNA concentration and quality were assessed using the Agilent High Sensitivity RNA ScreenTape System (Agilent, USA) following the provided protocol. Samples returned a mean RNA concentration of 2.5 ng μL^{-1} and mean RNA integrity number equivalent (RIN^e) score of 7.1. rRNA depletion was performed with the Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero Plus kit (Illumina, USA) following the manufacturer's instructions and sequencing carried out on the Illumina NovaSeq™ 6000 (Illumina, USA). Transcript abundance was quantified using Salmon for each gene in all samples. Differential gene expression was performed with DESeq2 in R software to quantify the $\log_2$ fold-change in transcript reads [120] for each gene and subsequent principal component analysis using DESeq2 and a built-in R method (prcomp) [43].

Cluster and gene ontology analyses

For cluster and gene ontology analysis, differential expression was tested with edgeR (version 3.28.1) [121]. Predicted log fold-changes were positively correlated between replicates, however a batch effect was detected, and replicate number was retained as a model term. Transcripts with low expression were filtered out of the data via edgeR before fitting the differential expression models. Clustering analysis was performed using the mclust package (version 5.4.7) for R [122]. Only transcripts with differential expression FDR < 0.05 in at least one cell type were subjected to clustering analysis. All variance structures were tested for a range of 2 to 20 clusters and the minimal, best-fitting model was identified by the Bayes information criterion. Gene ontology enrichment analysis was performed using the clusterProfiler package (version 4.10.0) for R [123,124]. Enrichment in terms belonging to the "Biological Process" ontology was calculated for each gene cluster, relative to the set of all genes quantified in the experiment, via a one-sided Fisher exact test (hypergeometric test). *P* values were adjusted for false discovery by using the method of Benjamini and Hochberg. Finally, the lists of significantly enriched terms were simplified to remove redundant terms, as assessed via their semantic similarity to other enriched terms, using clusterProfiler's simplify function.

Transcription factor activity

Relative activities of transcription factors were estimated from differential expression results (generated in edgeR) using VIPER (version 1.2.0) for R [51]. Transcription factor regulons were derived from the full RegulonDB database (version 10.9) [125], fixing the likelihood to 1. Only transcripts with differential expression FDR < 0.05 in at least one cell type were used to estimate transcription factor activities.

VIPER was run with a minimum allowed regulon size of 20 (*minsize=20*), on the full gene data set (*eset.filter=FALSE*) and was set to normalise enrichment scores (*nes=TRUE*).

Lipid extraction and lipidomic analysis

Lipid extractions were performed following a modified version of the Folch extraction [46,126]. Briefly, 100 µL of sample was added to 250 µL of methanol and 125 µL of chloroform in a microcentrifuge tube. Samples were incubated for 60 min and vortexed every 15 min. Then, 380 µL of chloroform and 90 µL of 0.2 M potassium chloride was added to each sample. Cells were centrifuged at 14,000 ×*g* for 10 min to obtain a lipophilic phase which was transferred to a glass vial and dried under a nitrogen stream.

The lipophilic phase was then resuspended in 20 µL of a methanol:chloroform solution (1:1 *v/v*), then 980 µL of an isopropanol:acetonitrile:water solution (2:1:1, *v/v/v*) was added. Samples were analysed on the Agilent 6560 Q-TOF-MS (Agilent, USA) coupled with the Agilent 1290 Infinity II LC system (Agilent, USA). An aliquot of 0.5 µL for positive ionisation mode and 2 µL for negative ionisation mode from each sample was injected into a Kinetex® 5 µm EVO C18 100 A, 150 mm × 2.1 µm column (Phenomenex, USA). The column was maintained at 50 °C at a flow rate of 0.3 mL/min. For the positive ionisation mode, the mobile phases consisted of (A) acetonitrile:water (2:3 *v/v*) with ammonium formate (10 mM) and B acetonitrile:isopropanol (1:9, *v/v*) with ammonium formate (10 mM). For the negative ionisation mode, the mobile phases consisted of (A) acetonitrile:water (2:3 *v/v*) with ammonium acetate (10 mM) and (B) acetonitrile:isopropanol (1:9, *v/v*) with ammonium acetate (10 mM). The chromatographic separation was obtained with the following gradient: 0-1 min 70% B; 1-3.5 min 86% B; 3.5-10 min 86% B; 10.1-17 min 100% B; 17.1-10 min 70% B. The mass spectrometry platform was equipped with an Agilent Jet Stream Technology Ion Source (Agilent, USA), which was operated in both positive and negative ion modes with the following parameters: gas temperature (200 °C); gas flow (nitrogen), 10 L min^{-1}; nebuliser gas (nitrogen), 50 psig; sheath gas temperature (300 °C); sheath gas flow, 12 L min^{-1}; capillary voltage 3500 V for positive, and 3000 V for negative; nozzle voltage 0 V; fragmentor 150 V; skimmer 65 V, octupole RF 7550 V; mass range, 40-1,700 *m/z*; capillary voltage, 3.5 kV; collision energy 20 eV in positive, and 25 eV in negative mode. MassHunter software (Agilent, USA) was used for instrument control.

Tachyplesin-NBD treated and untreated cells were analysed using UHPLC/Q-TOF-MS and detected a mean of 1,000 mass spectrometry features in positive and negative ionisation modes. Lipid annotation was performed following COSMOS consortium guidelines [127] with the CEU Mass Mediator version 3.0 online tool [128]. Lipid Annotator software (Agilent, USA) was then used to identify the lipid classes and their relative abundances (Figure 3E). Low and high tachyplesin-NBD accumulators and an untreated control sorted via fluorescence-activated cell sorting were analysed using Q-TOF-LC/MS. Mass Profiler 10.0 (Agilent, USA) was used to generate a matrix containing lipid features across all samples, then filtered by standard deviation, and normalised by sum calculations. Multivariate statistical analyses were performed on these features using SIMCA® software 15.0 (Sartorius, Germany). First, a principal component analysis was performed followed by a partial least square-discriminant analysis (PLS-DA) with its orthogonal extension (OPLS-DA), which was used to visualise differences between low and high tachyplesin-NBD accumulators and untreated control samples. Lipids with variable importance in projection (VIP) scores above 1 were annotated (Table S2).

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AUTHOR CONTRIBUTIONS

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COMPETING INTERESTS

The authors declare no competing interests.