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Citation

Omardien, S., Drijfhout, J. W., Veen, H. van, Schachtschabel, S., Riool, M., Hamoen, L. W., ... Zaat, S. A. J. (2018). Synthetic antimicrobial peptides delocalize membrane bound proteins thereby inducing a cell envelope stress response. *Bba - Biomembranes*, 1860(11), 2416-2427. doi:10.1016/j.bbamem.2018.06.005

Version: Not Applicable (or Unknown)

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Downloaded from: <https://hdl.handle.net/1887/86734>

Note: To cite this publication please use the final published version (if applicable).



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Contents lists available at ScienceDirect

BBA - Biomembranes

journal homepage: www.elsevier.com/locate/bbamem

Synthetic antimicrobial peptides delocalize membrane bound proteins thereby inducing a cell envelope stress response

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ARTICLE INFO

Keywords:

Amphipathic peptides
Cell envelope stress
Membrane bound proteins
Membrane perturbation
Thrombocidin-1 derivatives
BPI derivative

ABSTRACT

Background: Three amphipathic cationic antimicrobial peptides (AMPs) were characterized by determining their effect on Gram-positive bacteria using *Bacillus subtilis* strain 168 as a model organism. These peptides were TC19 and TC84, derivatives of thrombocidin-1 (TC-1), the major AMPs of human blood platelets, and Bactericidal Peptide 2 (BP2), a synthetic designer peptide based on human bactericidal permeability increasing protein (BPI). **Methods:** To elucidate the possible mode of action of the AMPs we performed a transcriptomic analysis using microarrays. Physiological analyses were performed using transmission electron microscopy (TEM), fluorescence microscopy and various *B. subtilis* mutants that produce essential membrane bound proteins fused to green fluorescent protein (GFP).

Results: The transcriptome analysis showed that the AMPs induced a cell envelope stress response (cell membrane and cell wall). The cell membrane stress response was confirmed with the physiological observations that TC19, TC84 and BP2 perturb the membrane of *B. subtilis*. Using *B. subtilis* mutants, we established that the cell wall stress response is due to the delocalization of essential membrane bound proteins involved in cell wall synthesis. Other essential membrane proteins, involved in cell membrane synthesis and metabolism, were also delocalized due to alterations caused by the AMPs.

Conclusions: We showed that peptides TC19, TC84 and BP2 perturb the membrane causing essential proteins to delocalize, thus preventing the possible repair of the cell envelope after the initial interference with the membrane.

General significance: These AMPs show potential for eventual clinical application against Gram-positive bacterial cells and merit further application-oriented investigation.

1. Introduction

Due to an increase in antimicrobial resistance development, effort has been placed on understanding the means by which bacteria acquire resistance and to search for new antimicrobials. To address the latter, antimicrobial peptides (AMPs) have been proposed as a potential novel class of antibiotics [1, 2]. AMPs are thought to reduce the chances of resistance development due to their non-specific rapid membrane targeting effect [2]. However, the non-specific activity of AMPs can cause an increase in toxicity to mammalian cells [1, 2]. Additionally, AMPs have a low bioavailability, are prone to protease degradation, and the

production cost is higher than for classical antimicrobials [1, 2]. Furthermore, reports have been made of pathogenic bacteria that have shown to develop resistance against natural AMPs [3]. Thus, rational design of AMPs has been employed with the intention of developing cost effective highly active short peptides with simple structures. Naturally occurring peptides or proteins are used as a starting point for peptide design, for example peptidomimetic POL7080 derived from protegrin I (PG-I) [4], semi-synthetic NVB302 derived from deoxyactagardine B [5], P113 derived from histatin 5 [6] and Omiganan derived from indolicidin [7].

To understand the distinctive attributes of AMPs that lead to their

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efficient antimicrobial activity, efforts are placed in determining the mode of action of designed peptides. For this study, peptides TC19 and TC84, derived from thrombocidin-1 (TC-1) [8], and the designer peptide BP2 [9] were selected. Peptide TC19 and TC84 were derived from the N-terminal end of TC-1 and were modified to improve their antimicrobial activity compared to the native peptide [8]. TC19 and TC84 only differ by one amino acid, i.e. a cysteine (C) was replaced by an alanine (A) near the C-terminal end, resulting in an increase in stability of TC84 in 100% human plasma compared to TC19 [8]. Both TC19 and TC84 showed antimicrobial activity against *Staphylococcus aureus* [8]. Peptide TC19 further showed to be a broad spectrum antimicrobial by being active against *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and an extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* [8]. TC19 has antifungal activity against *Aspergillus niger* and *Candida albicans*, and has anti-biofilm activity against *S. aureus* [8]. BP2 was fashioned through molecular modelling and rational design based on the LPS-binding domains of bactericidal permeability increasing protein (BPI) [9]. BP2 has shown to be active against both Gram-positive and -negative bacteria [9–11].

All three peptides are predicted to share common features such as their amphipathic nature and cationic charge but differ in amino acid sequence. Noticeably, TC84 contained one alpha-helix promoting alanine residue [12, 13] and BP2 two. In this study, we aimed to determine the mode of action of TC19, TC84 and BP2 using the Gram-positive bacterium *Bacillus subtilis*. We expected to observe a related stress response between the TC peptides, but different from the response seen upon exposure to BP2. We found TC19, TC84 and BP2 to be active in the micro-molar range against *B. subtilis*. Our transcriptomic analysis showed that both the cell membrane and cell wall were targeted, which we confirmed during the physiological observations. We found that TC19, TC84 and BP2 perturbed the membrane of *B. subtilis* cells in a concentration dependent manner without directly damaging the cell wall. This led to the delocalization of essential proteins involved in cell wall synthesis, cell membrane synthesis and metabolism contributing to the envelope stress. Minor differences in the stress response between the TC peptides and the BP2 peptide were observed.

2. Materials and methods

2.1. AMP information, strains used and the culturing conditions

TC19 (LRCMCIKWWSGKHPK), TC84 (LRAMCIKWWSGKHPK) and BP2 (GKWKLFKKAFKFLKILAC) were dissolved in 0.01% acetic acid and stored at -20°C . Stocks were thawed on ice prior to experiments.

Bacillus subtilis strains used in the study can be found in Table S1. *B. subtilis* cultures were prepared in complete minimal medium (CMM). This medium contained Spizizen's Minimal Medium (SMM), as described in Anagnostopoulos & Spizizen (1961) [14], with the modifications described in Halbedel et al. (2014) [15]. Pre-cultures were prepared by inoculating a single colony from Luria Broth (LB) solid medium into 5 ml LB medium and culturing overnight. The overnight culture was inoculated into CMM or LB to have an initial optical density at an absorbance of 600 nm (OD_{600}) of 0.05 and subsequently incubated until an OD_{600} of 0.4 to 0.6 (the early exponential growth phase) was obtained. The pre-cultures were diluted for each experiment to an OD_{600} of 0.2, if not specified otherwise. Culturing was performed at 37°C under continuous agitation at 200 rpm where appropriate. Culturing media were supplemented when required. Information about the medium supplements required for each strain can be found in the Table S1.

2.2. Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

To obtain the lowest concentration necessary to have an inhibitory effect on *B. subtilis* (strain 168), the MIC was determined. The MBC was

determined to establish whether the AMPs are lethal at concentrations close to the MIC. The MIC was determined by measuring the OD_{600} for 24 h in a microtiter plate reader (Multiskan FC, Thermo Scientific). *B. subtilis* cells at an OD_{600} of 0.02 (1×10^7 CFU/ml) were treated in CMM containing the AMP. A two-fold serial dilution from 56 μM to 0.11 μM of the AMP was prepared in a final reaction volume of 150 μl in each well. The control consisted of CMM without AMP. The experimental conditions to determine the MBC were similar to the MIC, but after 24 h the culture in every well was plated out onto LB solid medium. The MIC was considered to be the lowest AMP concentration where no outgrowth was observed, meaning no change in OD_{600} for *B. subtilis* cells. The MBC was considered to be the lowest AMP concentration which killed 99.99% of the culture after 24 h. The Student's *t*-test was applied to determine statistical significance between two groups.

2.3. Assessing the development of resistance to the AMPs

Adaptation or the development of resistance was evaluated by performing an evolutionary study. *B. subtilis* was inoculated at an OD_{600} of 0.02 (1×10^7 CFU/ml) in CMM with AMPs ranging from 56 μM to 0.44 μM . Culturing was performed for 24 h and the MICs were determined by measuring the OD_{600} . The lowest concentration that had no detectable growth was noted as the MIC. Cultures that had an OD_{600} of $\geq 50\%$ of the OD_{600} of the untreated cultures were selected to be re-inoculated in fresh CMM containing the AMPs. Cultures were inoculated into the fresh medium to a final OD_{600} of 0.02. Cultures were subjected to repeated exposure to the AMPs for 14 passages. Ciprofloxacin was used as a positive control. Ciprofloxacin is an antibiotic of the fluoroquinolone class known to target DNA gyrase and topoisomerase IV, thus inhibiting DNA replication [16]. Results were reported as fold change in MIC over number of passages.

2.4. Time-kill assay to observe the killing effect of the AMPs on *B. subtilis* at concentrations close to the MIC values

To observe the killing effect of the AMPs on *B. subtilis* cells over time, a time-kill assay was performed using the MIC concentrations determined previously. The cell number for the time-kill assay was increased from 1×10^7 CFU/ml, used during determining the MIC, to 1×10^8 CFU/ml. A pre-culture was diluted to an OD_{600} of 0.2 (1×10^8 CFU/ml) with CMM and divided into 1 ml aliquots. To each 1 ml aliquot, the AMP was added to reach a final concentration at the MIC and at values close to the MIC. This was 56 μM to 0.11 μM for TC19, TC84 and BP2. The control was a culture incubated without AMPs. From each 1 ml reaction, 25 μl was removed and added to 25 μl of 0.1% w/v polyanetholesulfonic acid sodium salt (SPS), a polyanionic polymer that neutralizes the cationic AMPs [10, 17]. The samples in SPS were diluted in 0.85% w/v NaCl (saline solution) to obtain a ten-fold serial dilution range from 10^{-1} to 10^{-6} . From the undiluted sample and dilution series, 10 μl was removed and spotted onto LB solid medium. The overnight incubation was performed at 37°C . The number of CFUs were quantified and the results were expressed as \log_{10} CFU/ml. Aliquots were removed at time points 5, 30, 60 and 120 min. Three biological repeats were performed. Concentrations used in subsequent experiments are based on the time-kill assay results.

2.5. Preparation of RNA for microarray analysis

The sample preparation for RNA isolation was performed by culturing *B. subtilis* overnight in 200 ml CMM. The overnight culture was diluted with pre-warmed CMM to have a final OD_{600} of 0.02 and volume of 600 ml. When the culture reached an OD_{600} of 0.4, it was diluted with pre-warmed CMM to have a final OD_{600} of 0.2. The culture was split into 150 ml fractions and added to 1 l polypropylene Erlenmeyer flasks. To each flask AMP was added to have a final concentration of 3.5 μM TC19 and TC84, and 0.22 μM BP2. These peptide

concentrations caused a one log reduction in CFU/ml during the time kill assay. The cultures were incubated for 5 min and 120 min. SPS was added to have a final concentration of 0.05% w/v and the cells harvested by centrifuging for 2 min at 10,000 rpm and at 20 °C using the Sorvall RC-6 (Thermo Scientific). Residual medium was removed by centrifuging the pellet for 5 min at 4000 rpm. The pellet was snap frozen using liquid nitrogen for subsequent RNA isolation.

RNA was isolated by initially grinding the frozen pellet with a mortar and pestle. The ground cells were added to occupy about 50 µl of a pre-chilled Eppendorf tube. To each Eppendorf tube, 300 µl Trizol was added. After incubating the mixture for 5 min at room temperature (20 °C), 60 µl chloroform was added and incubation at room temperature was continued for 3 min. The mixture was centrifuged for 15 min at 12,000 rpm at 4 °C and the upper aqueous layer removed. This aqueous layer was added to 1 volume 70% ethanol and the mixture transferred to a RNeasy MinElute spin column (Qiagen). Subsequently, the method prescribed by the manufacturer of the RNeasy Mini Kit (Qiagen) was followed.

2.6. Synthesis of labelled cDNA, hybridization, and scanning of the DNA microarrays

The RNA concentrations were measured on the NanoDrop ND-2000 (Thermo Scientific). The integrity of the RNA samples was assessed on a 2200 TapeStation system (Agilent Technologies) using the RNA ScreenTape (Agilent Technologies). Per sample, 5 µg of total RNA was combined with ArrayControl RNA Spikes (Ambion) and 1 µg random octamers (Biolegio), denatured at 65 °C for 10 min and placed on ice-water for 5 min. Subsequently, a first strand master mix was added containing first strand buffer (Thermo Fisher Scientific), 0.5 mM dGAC, 0.35 mM dUTP, 0.15 mM dUTP-Cy3 (GE Healthcare) and 200 U SuperScript IV (Thermo Fisher Scientific). This mixture was subsequently incubated for 5 min at 25 °C, 60 min at 50 °C and 10 min at 80 °C. Finally, NaOH was added to hydrolyse the remaining RNA by heating at 70 °C for 15 min. The reaction was stopped by adding MOPS buffer and the labelled cDNA was purified with the E.Z.N.A. MicroElute RNA Clean-up Kit (Omega Biotek). Dye incorporation and cDNA yield were measured on the NanoDrop ND-2000 yielding a frequency of incorporation of > 10 pmol/µg.

Each hybridization mixture was made up from 1.1 µg Test (Cy3) and 1.1 µg Reference (Cy5) sample. Samples were dried and 1.98 µl water was added. The hybridization cocktail was made according to the manufacturer's instructions (NimbleGen Arrays User's Guide — Gene Expression Arrays Version 5.0, Roche NimbleGen). To each sample 7.2 µl from this mix was added. The samples were incubated for 5 min at 65 °C and 5 min at 42 °C prior to loading. Hybridization samples were loaded onto a 12 × 135 K microarray custom designed against *B. subtilis* (Roche NimbleGen). Microarrays were hybridized for 20 h at 42 °C with the NimbleGen Hybridization System (Roche NimbleGen). Afterwards, the slides were washed according to the NimbleGen Arrays User's Guide — Gene Expression Arrays Version 6.0 and scanned with an Agilent DNA microarray scanner G2565CA (Agilent Technologies). Feature extraction was performed with NimbleScan v2.6 (Roche NimbleGen).

2.7. Microarray data extraction and processing

The microarray data were analysed using the R statistical language (<https://cran.r-project.org/>) with packages made available by the Bioconductor project (<https://www.bioconductor.org/>). Gene expression values were calculated using the robust multi-array average (RMA) algorithm [18]. The normalized data was statistically analysed for differential gene expression using a mixed linear model with coefficients for Block (random), and each experimental treatment (fixed) [19, 20]. A contrast analysis was applied to compare each exposure, which were 5 or 120 min of treatment with the peptide, with the control, which was the untreated sample. The F_s test statistic [21] was used for

hypothesis testing and the resulting *p*-values were corrected for false discoveries according to [22]. Genes were considered to be differentially expressed when the expression ratio exceeded a factor of two and showed a significant difference in log expression ratio (*p* ≤ 0.05). Identified genes were categorized according to SubtiWiki (<http://subtiwiki.uni-goettingen.de/>). The hierarchical clustering was performed using the Euclidean distance method and complete agglomeration method.

2.8. Confirming membrane damage using transmission electron microscopy (TEM)

Physiological changes to *B. subtilis* after treatment were observed using TEM. The culture was prepared as mentioned previously in the time-kill assay and harvested after incubating the culture with the AMPs for 5 or 120 min. One volume of Mc Dowell's fixative was added to the treated culture and the cells were pelleted using centrifugation for 2 min at 10,000 rpm. The supernatant, containing the fixative and medium, was removed and undiluted Mc Dowell's fixative added to the pelleted cells for preservation until further processing.

Negative staining of the cells was performed using uranyl acetate. In brief, the Mc Dowell's fixative was removed from the sample after centrifugation. The cells were washed once and re-suspended with double distilled water. Carbon coated grids were placed on top of a 10 µl aliquot of bacterial cell suspension for 2 min and washed 5 times on a drop of distilled water. The grids were, subsequently, placed on top of a small drop of 3.5% uranyl acetate for 1.5 min and excess uranyl acetate was removed by holding the grids to a filter paper at an angle of 45°. The grids were finally dried in the petri dish with filter paper prior to imaging.

The *B. subtilis* cells on the grids were visualized and examined with a FEI Technai-12 Spirit Biotwin transmission electron microscope (FEI, Eindhoven, Netherlands) and micrographs were taken with a Veleta side-mounted TEM camera using Radius acquisition software (Olympus Soft Imaging Solutions, Münster, Germany). Image measurements were performed with processing features within the Radius software package. One biological repeat was performed.

2.9. Membrane perturbation measurement using the fluorescent dye, Sytox Green

Sytox Green (Molecular Probes, Invitrogen), a nucleic acid staining dye that can only penetrate a compromised membrane, was used to determine whether the AMPs caused membrane perturbation. Flow cytometry was employed to quantify the number of cells positively stained with Sytox Green after treatment with the AMPs for 5 min. *B. subtilis* cells were pelleted using centrifugation at 10000 rpm for 2 min and residual CMM removed. The pellet was re-suspended in 0.85% w/v NaCl and 5 µM of Sytox Green (Molecular Probes, Invitrogen) added. The mixture was incubated for 15 min in the dark at room temperature. The stained cells were subsequently washed twice with 0.85% w/v NaCl and re-suspended in 1 ml 0.85% w/v NaCl. Stained and unstained cells were counted based on fluorescence measurements after exposure to an argon lamp (488 nm) at an excitation and emission wavelength of 500 to 550 nm, respectively, using the Gallios Flow cytometer (Beckman Coulter). A total of 10,000 cells were counted and the un-gated results were reported as % Sytox Green stained cells. Three biological repeats were performed. Microscopy imaging was performed using the Olympus BX-60. The Olympus BX-60 was mounted with a CoolSnap fx (Photometrics) CCD camera and an UPLANFL 100 × /1.3 oil objective (Tokyo, Japan). Microscopy images were analysed in ImageJ (<http://rbsweb.nih.gov/ij/>).

2.10. Bacterial cytological profiling using mutants producing green fluorescent protein (GFP) fusion proteins

B. subtilis mutants expressing proteins fused to the GFP, were used to determine whether the AMPs caused delocalization of proteins involved in various cellular processes, thus rendering these processes inactive. Culturing was in CMM containing the required supplements for induction (Table S1). Treatment with the AMPs was for 5 min while shaking at 37 °C. GFP-fused proteins were visualized using the Nikon Eclipse Ti fluorescence microscope at an excitation wavelength of 395 ± 5 nm and emission wavelength of 509 ± 5 nm. Microscopy slides were prepared by transferring 0.5 µl culture onto a thin 1.5% w/v agarose pad on a microscopy slide. These experiments were performed in duplicate. Quantification of images were performed by counting 200 cells of three biological repeats and expressing the results as % cells with delocalized proteins of the total cells counted. The Nikon Eclipse Ti was equipped with an Intensilight HG 130 W lamp, a C11440-22CU Hamamatsu ORCA camera, a CFI Plan Apochromat DM 100× oil objective, an OkoLab stage incubator (Napoli, Italy) and with the NIS elements software version 4.20.01. Microscopy images were analysed in ImageJ/Fiji (<http://rbsweb.nih.gov/ij/>).

3. Results

3.1. Differences in antimicrobial activity between AMPs are marginal

The MIC and MBC were determined to observe possible differences in activity and to obtain the lowest peptide concentrations necessary to evaluate the mode of action of the AMPs against *B. subtilis* strain 168. The difference between the MIC and MBC values of the AMPs against *B. subtilis* were not significant (Table 1).

3.2. *B. subtilis* does not adapt or develop resistance to the AMPs

To determine whether *B. subtilis* adapts to or develops resistance against TC19, TC84 and BP2, *B. subtilis* was passaged 14 times through various concentrations of TC19, TC84 and BP2. Ciprofloxacin, a fluoroquinolone that inhibits DNA gyrase, was tested as a positive control for resistance development. Whereas *B. subtilis* developed resistance to ciprofloxacin within 10 passages, the strain was unable to grow at a concentration higher than two-fold of the MIC of the peptides, showing that adaptation or resistance does not occur against TC19, TC84 or BP2 throughout the 14 passages of the experiment (Fig. 1).

3.3. TC19, TC84 and BP2 kill cells rapidly

The time-kill assay for TC19, TC84 and BP2 against *B. subtilis* cells showed a rapid decline in numbers of colony forming units (CFU) at 5 min at concentrations of 56 µM to 7 µM for TC19 and TC84, and 3.5 µM to 0.44 µM for BP2 (Fig. 2) against a cell number 10-fold higher than what was used during the MIC/MBC measurements. Concentrations higher than 3.5 µM BP2 also caused rapid killing and are not shown. Peptides TC19 and TC84, however, caused a further decline in

Table 1

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the antimicrobial peptides against *B. subtilis* strain 168 (1.5×10^6 cells/well).

Antimicrobial peptide	MIC (µM)	MBC (µM)
	Mean ± SD	Mean ± SD
TC19	7.0 ± 3.3 (n = 6)	7.0 ± 4.6 (n = 3)
TC84	7.0 ± 4.2 (n = 6)	3.5 ± 0.2 (n = 3)
BP2	3.2 ± 1.5 (n = 6)	3.5 ± 0.2 (n = 3)

n is the number of biological repeats performed.

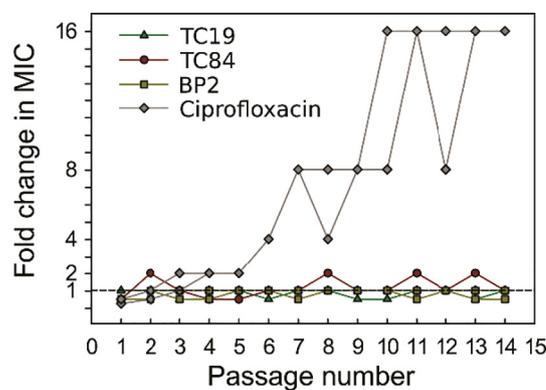


Fig. 1. Adaptation or resistance development of *B. subtilis* after TC19, TC84 and BP2 treatment using an evolutionary study. Ciprofloxacin was used as a positive control. Induction of adaptation or resistance was performed by repeated exposure of *B. subtilis* to ¼MIC, ½MIC, 1 × MIC, 2 × MIC and 4 × MIC, of TC19, TC84 and BP2 for 14 passages. The lowest concentration that had no detectable growth after these 14 passages was reported as fold change of the MIC. The highest detectable fold change observed for each of the peptides was two, which was considered not to be adaptation or resistance development. An inoculum of 1×10^7 CFU/ml was used and culturing was performed for 24 h. Two biological repeats are shown.

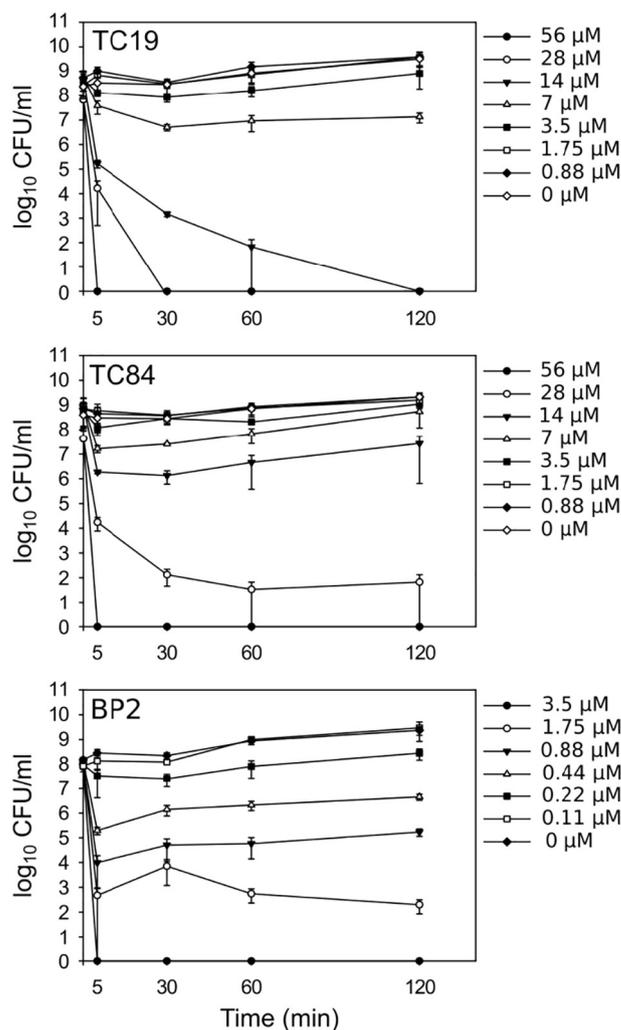


Fig. 2. Time-kill curves depicting the numbers of *B. subtilis* surviving cells after TC19, TC84 and BP2 treatment.

CFU upon 30 min of incubation. TC19 showed a further decline at 14 μM after 60 min, followed by a complete killing of the culture. BP2 only showed a decline at 5 min with no bacteria surviving at 3.5 μM . TC19 and TC84 are thus slower acting compared to BP2. This difference could be due to the difference in net positive charge of TC19 and TC84, which is 4+, compared to that of BP2 which is 7+. Thus, the electrostatic interaction of BP2 to the net negatively charged cell envelope of *B. subtilis* might be greater in the case of BP2 than with TC19 and TC84. Survival of remaining cells in the incubation could occur due to the reduction of available peptide in the medium, due to protease degradation or due to binding of the peptides to cells, cell debris, components of the medium or to the surface of the microtiter wells (Fig. S1). Concentrations used in the subsequent experiments were based on the time kill assay results.

Aliquots of the culture were taken at 0, 5, 30, 60 and 120 min. Surviving cells were expressed as colony forming units (CFU) per ml. Standard error bars represent three biological repeats. CFU/ml values that were zero were substituted with one, to display the values on the graph.

3.4. *B. subtilis* transcriptional response after treatment with AMPs reveals cell envelope stress

To elucidate the mode of action of TC19, TC84 and BP2, we first performed a transcriptomic analysis of *B. subtilis* cells treated with sub-lethal concentrations at 5 and 120 min after peptide addition. *B. subtilis* differentially expressed the highest number of genes in response to TC19 (Table 2). The majority of the genes differentially expressed after treatment with TC84 were also expressed in response to TC19 (Fig. 3). Five minutes of treatment with BP2 yielded the lowest number of differentially expressed genes, and most of these genes were also differentially expressed in response to TC19 and/or TC84 (Fig. 3). No genes were differentially expressed after 120 min treatment with BP2. The stress response of TC19, TC84 and BP2 was different from that of known antimicrobials that target cell wall synthesis (D-cycloserine, oxacillin, ristocetin, bacitracin, vancomycin and amoxicillin), the cell membrane (the non-ionic detergent Triton X-114), the cell membrane without permeabilising the membrane (the ionophore monensin) and compounds that target the cell membrane by permeabilising the membrane (gramicidin A and polymyxin B) [23] (Fig. 4). The data show that the stress response of the AMPs was most similar to that of the detergent, Triton X-114. We chose in our analysis to focus on specific regulons to obtain an overview of the response of *B. subtilis* to the AMPs. Only key genes that indicate a possible mode of action of the peptides are mentioned. Individual genes might not be differentially expressed by all three peptides.

B. subtilis responded to TC19, TC84 or BP2 by upregulating genes associated with the following two-component systems (TCSs) and their cognate genes: the *liaH-liaGFSR* operon, the *bceRS-bceAB* TCS-ABC transporters, the *psdRS-psdAB* TCS-ABC transporters, the *ycdJKLMyxeA* operon, *ytrABCDEF* and *ywoBCD* operons, and the *yvrHb* regulon (Fig. S2 and Table S2). *B. subtilis* induced the expression of genes involved in all of the TCS mentioned in response to a 5 min TC19 exposure. Only the genes associated with the BceRS TCS, the YtrA regulon, the PsdRS

Table 2

The number of differentially^a expressed genes after treatment with the AMPs.

	TC19		TC84		BP2	
	5 min	120 min	5 min	120 min	5 min	120 min
Upregulated	187	528	107	36	39	0
Downregulated	7	470	1	4	0	0

^a Genes are considered to be differentially expressed when the expression ratio exceeds a factor of two and shows a significant difference in log expression ratio ($p \leq 0.05$).

Comparison of total differentially expressed genes

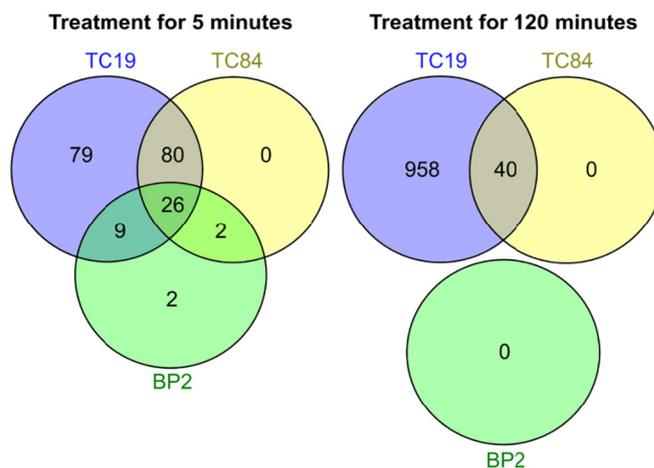


Fig. 3. Venn diagrams depicting the numbers of shared differentially expressed genes in response to TC19, TC84 and BP2.

TCS and the YvrHb regulon were differentially upregulated in response to 120 min of treatment with TC19. In response to 5 min of treatment with TC84, genes associated with the LiaRS TCS, BceRS TCS, YxdJK TCS, the YtrA regulator and the YvrHb TCS were responsive. These genes were not upregulated in response to 5 min of treatment with BP2, except for one gene in the *ytrABCDEF* operon, and two in the YvrHb TCS. None of the TCSs and their cognate genes were differentially upregulated after 120 min of treatment with TC84 or BP2. When comparing the stress response with the above-mentioned antimicrobials, only Triton X-114, bacitracin and vancomycin exposed cells showed an induction of expression of the genes associated with the LiaRS TCS [23]. Furthermore, genes associated with the BceRS TCS were only induced by bacitracin and ristocetin, and those associated with the YtrA regulator by vancomycin and ristocetin [23].

The LiaRS TCS is upregulated in response to cell wall synthesis inhibition but also to membrane perturbation [24–29]. The BceRS TCS, PsdRS TCS and the YtrA regulon are upregulated during cell wall synthesis inhibition [24, 25, 30–32]. YxdJK TCS are associated with cell membrane perturbation [26]. The YvrHb regulon plays a key role in maintaining the cell envelope integrity by positively regulating *wprA*, *wapA-yxxG*, *dltABCDE*, *sunA*, *sunT-bdbA-yoJ-bdbB*, *yvrI-yvrHa* and *sigX-rsIX*, and negatively regulating the *lytABC* operon [33, 34].

B. subtilis responded to TC19, TC84 or BP2 by upregulating the expression of genes under control of the extracytoplasmic function (ECF) sigma factors SigM, V, W and X, which play a key role in cell envelope stress response [24–26, 31, 35–44] (Fig. S2). Genes associated with SigB were also differentially expressed, but most of the genes upregulated after 5 min and 120 min are shared by SigM, W or X (Fig. S3). Downregulated genes associated with SigB were mostly differentially expressed after treatment with TC19 for 120 min, and the majority of these genes were not regulated by SigM, W or X (Table S3). These genes were involved in multidrug resistance and resistance against ethanol, salt, paraquat, low temperature, peroxide, and other antimicrobial stimuli (Table S3).

For an overview of the genes regulated and differentially expressed by SigM, V, W and X in response to TC19, TC84 or BP2 refer to Table S4. Results on genes relevant for the mode of action are summarized as follows. Genes involved in cell wall stress were upregulated. These were *ydaH* (also *amj*), *bcrC*, the penicillin-binding protein genes *pbpX* and *pbpE*, and *ywaC*, the biomarker gene for screening cell-wall active compounds [45]. Similarly, Triton X-114 induced the expression of *pbpE*, amoxicillin the *ywaC*, vancomycin the *ydaH*, and ristocetin the *bcrC* and *ywaC* [23]. The *ydaH* gene encodes a lipid II flippase involved in the transport of lipid II across the membrane [46] and *bcrC* encodes

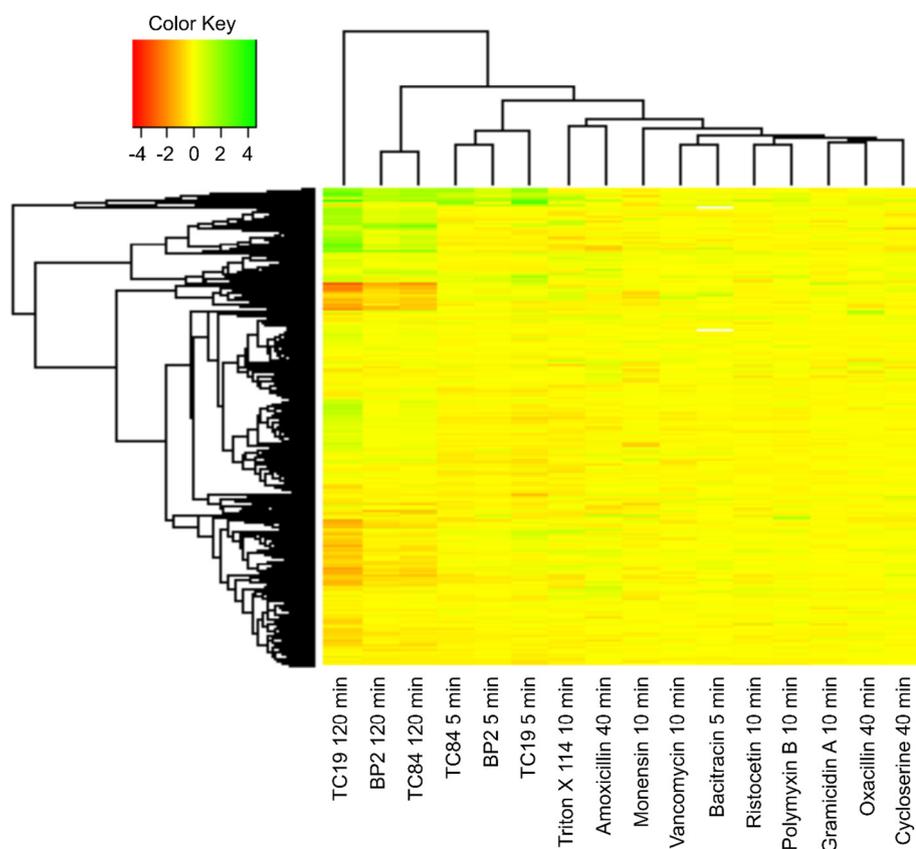


Fig. 4. Hierarchical clustering analysis of gene expression in response to TC19, TC84, BP2 and other cell envelope-active antibiotics. Antibiotic treatments included were cell wall synthesis inhibitory antimicrobials (D -cycloserine, oxacillin, ristocetin, bacitracin, vancomycin and amoxicillin), a detergent (Triton X-114), a cell membrane active antimicrobial that does not permeabilise the membrane (the ionophore monensin) and those that do permeabilise the membrane (gramicidin A and polymyxin B) [23]. Gene expression levels are shown by colour according to the colour key, red indicating down-regulation and green indicating upregulation.

undecaprenyl pyrophosphate phosphatase important for cell wall synthesis and is involved in resistance against bacitracin and paraquat [31, 47, 48]. Genes involved in cell envelope biogenesis (*divIB*, *mreC*, *mreD*, *murB* and *rodA*), and lipoteichoic acid synthesis (*yfnI*) were also upregulated [49–54]. These responses indicate that the peptides impose cell wall stress on the *B. subtilis* cells.

B. subtilis also responded to TC19 and TC84 by upregulating genes involved in modifying the cell surface such as the *dlt* operon, *psd*, and *oat*. *DltA*, *DltB*, *DltC*, *DltD* and *DltE* are all involved in the D-alanylation of teichoic acids and lipoteichoic acids [55]. *Psd* is a phosphatidylserine decarboxylase and is involved in the synthesis of phosphatidylethanolamine (PE), a zwitterionic phospholipid of the cell membrane [56]. *Oat* is involved in *O*-acetylation of peptidoglycan, a modification that has shown to be involved in resistance of Gram-positive bacteria against lysozyme [57].

Fatty acid metabolism was increased in response to exposure of *B. subtilis* cells to TC19 by the upregulation of *yrhJ*, a cytochrome P450 monooxygenase (CYP) referred to as P450 CYP102A3 for *B. subtilis* [58–60] (Table S4). CYP102A3 hydroxylates branched chain fatty acids, and the degradation of iso and anteiso fatty acids by CYP102A3 has been proposed as a means of altering the fluidity of the cell membrane [60, 61]. *B. subtilis* also upregulated *floT*, *floA*, *yuaF* and *pspA* in response to TC19 and TC84. Genes *floT*, *floA* and *yuaF* are involved in membrane fluidity homeostasis and *B. subtilis* can alter its membrane fluidity by upregulating these genes. The phage shock homologue and biomarker for membrane distortion, *PspA*, is involved in stabilizing the membrane [40, 62, 63]. These responses all indicate a response of the bacteria to cell membrane distortion by the peptides. Interestingly, only treatment with Triton X-114 showed an upregulation of the expression of *yuaF* and *pspA* [23].

Bacterial cells may experience oxidative stress and DNA damage due to cell envelope distortion after treatment with the peptides. Indeed we found *bcrC*, *yqjL*, *spx* and *yjbC* upregulated, which are associated with oxidative stress or paraquat resistance [47, 64–69]. Similarly, *yqjL* was

upregulated by Triton X-114, amoxicillin, vancomycin, ristocetin, and polymyxin B, and *yjbC* by amoxicillin and ristocetin. Transcription of a DNA repair gene, *recU* [70], and DNA integrity scanning gene, *disA*, [71] was upregulated. The sigma factor Xpf was differentially upregulated after treatment with TC19 and TC84, but not after treatment with BP2. *B. subtilis* has a suicidal response to DNA-damage by lysing and by producing the phage-like bacteriocin PBSX bacteriophage particles that kill strains non-lysogenic for this phage by damaging the cell wall [72]. Xpf positively regulates the expression of genes associated with PBSX particles production [72]. However, genes regulated by the Xre regulon were also upregulated after treatment with TC19 and TC84. Xre is a transcriptional repressor of Xpf [72]. The combined effects of upregulation of Xpf and Xre are unclear.

Finally, the genes *fosB*, *ybfO*, *ydbS*, *ydbT*, *yqeZ*, *yqfB*, *sunI*, *yfhL* and *yknWXYZ* reported to be involved in resistance to antimicrobials were upregulated (Table S5). *FosB* is a metallothiol transferase shown to confer resistance to fosfomycin and to the antimicrobials produced by *B. amyloliquefaciens* [35, 36]. *YbfO* is similar to an erythromycin esterase known to be involved in erythromycin resistance [32]. Genes *ydbS* and *ydbT* are involved in the resistance against the antimicrobials produced by *B. amyloliquefaciens* [35]. Genes *yqeZ* and *yqfB* as part of the *yqeZyqfAB* operon [35] and *sunI* [73] are involved in sublancin resistance, and *yfhL* and the *yknWXYZ* operon in resistance to the *B. subtilis* toxic protein SdpC [35]. The gene encoding SdpC, *yvaY*, was not differentially expressed. However, mutants with deletions of genes (ones that are non-essential) mentioned above did not show reduced susceptibility to TC19, TC84 and BP2 (Table S6). Interestingly, Triton X-114 induced the expression of *fosB*, *ybfO*, *ydbS*, *yqeZ*, *yqfB*, *yfhL*, *yknWXYZ*, but none of the other antimicrobials [23].

In summary, the transcriptomic analysis suggested that *B. subtilis* responded to the TC19, TC84 and BP2 exposure by upregulating genes associated with cell membrane distortion and cell wall synthesis. The induction of other gene sets indicates that alteration to the cell envelope likely caused oxidative stress and possibly DNA damage. *B. subtilis* thus

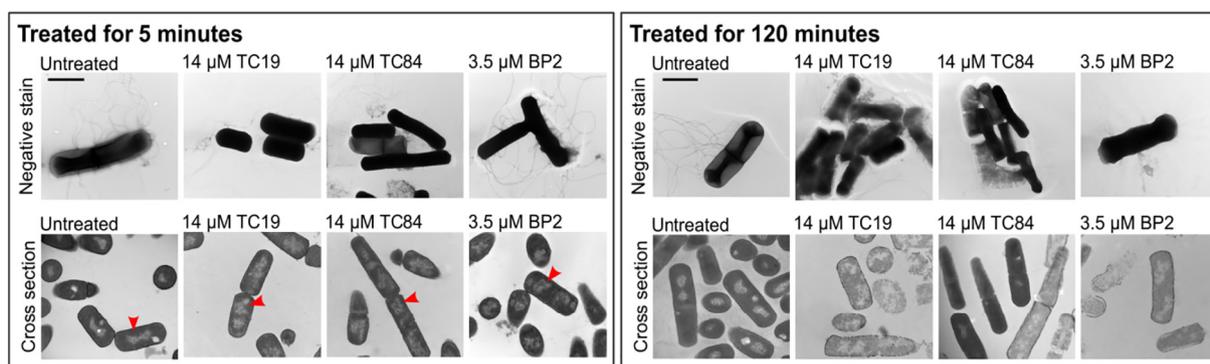


Fig. 5. Transmission electron micrographs (TEM) of *B. subtilis* after peptide treatment. *B. subtilis* was treated for 5 and 120 min with 14 μM TC19, 14 μM TC84 or 3.5 μM BP2. Negative staining with uranyl acetate was performed of cells after treatment with the peptides and cross sections were performed of the uranyl acetate stained cells. Negative staining showed cells stained black with uranyl acetate indicating perturbed membranes (treated). Cells with an intact membrane appears grey with black uranyl acetate on the surface of the cell (untreated). Cross sections of the stained cells after treatment for 5 min with TC19, TC84 and BP2 had irregular nucleoids compared to the untreated cells (red arrows). After 120 min of treatment with the peptides the cross sections reveal lysed cells, while all observed untreated cells remained intact. Scale bar of microscopy images represent 1 μm .

respond to the changes to the cell envelope by gene expression aimed at changing the cell surface, altering membrane fluidity and upregulating genes associated with resistance against known antimicrobials.

3.5. TC19, TC84 and BP2 cause membrane damage

Since the transcriptomic analysis suggested that the cell envelope is targeted by the peptides, we employed TEM to investigate any structural changes that the treatment of TC19, TC84 and BP2 might create after 5 min and 120 min. Lethal concentrations were used, which were 14 μM TC19 and TC84, and 3.5 μM BP2. Cells treated for 5 min with TC19, TC84 and BP2 stained black with uranyl acetate whereas the untreated cells appeared grey with black uranyl acetate deposited on the surface of the cell (Fig. 5). TC19, TC84 and BP2 did not lyse the cells and no visible cell wall damage was observed at 5 min. In contrast, after 120 min of exposure, disrupted cells were observed (Fig. 5). Cross sections of the cells treated for 5 min showed an irregularly distributed and abnormal nucleoid (Fig. 5, red arrows). Uranyl acetate staining of the cells entirely, implying that membrane perturbation had occurred since the membrane was permeable to the dye. Membrane permeabilisation, in a peptide concentration dependent manner, was confirmed using the fluorescent dye Sytox Green (Fig. 6). These findings suggest that the cell membrane is the primary target for the peptides. The cell wall stress response might be a result of cell membrane distortion and

the irregular nucleoid could be a consequence of the loss of cell membrane integrity.

3.6. Delocalization of membrane bound proteins

To further address the molecular events involved in cell envelope homeostasis we analysed in more detail protein localization in the cell membrane starting from the notion that essential cellular processes, such as cell wall and cell membrane synthesis, occur at the plasma membrane [74]. To study the localization of various proteins involved and to aid in the elucidation of their function, we used a panel of mutant strains expressing proteins fused to green fluorescent protein (GFP), to investigate whether the membrane proteins delocalized after treatment with TC19, TC84 and BP2. The *B. subtilis* mutants were cultured in defined minimal medium (CMM), in which the growth of the bacteria is slow and protein localization might differ from previous reports where rich medium was used (Fig. S4). Defined minimal medium was selected as the exact concentration of each component is known, unlike rich medium.

MinD was initially evaluated, where MinD together with MinC forms a complex that inhibits the Z-ring formation [75]. MinD-GFP is known to localize at the septum and at the cell poles [76]. In non-treated cells (Fig. 7, image a) MinD-GFP indeed was localized at the septum, whereas once TC19, TC84 and BP2 were added MinD-GFP

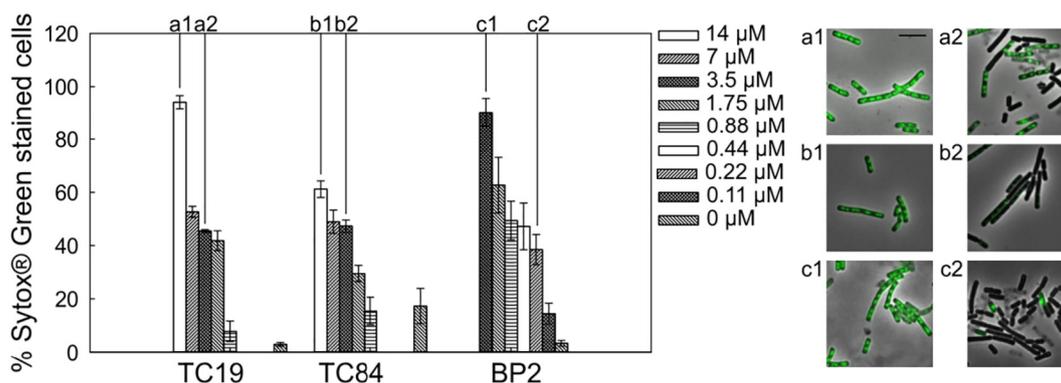


Fig. 6. Sytox Green staining of *B. subtilis* after peptide treatment. Sytox Green stained cells were visualized at the green emission wavelength with fluorescence microscopy and quantified with flow cytometry. At a high concentration of AMP, 14 μM of TC19 and TC84, and 3.5 μM BP2, membrane perturbation can be observed as Sytox Green staining of the nucleic acid is visible in image a1, b1 and c1. Membrane perturbation was also detected at a lower concentration of AMP, at 3.5 μM TC19 and 0.22 μM BP2 as shown in images a2, b2 and c2. Peptide TC84 at a low concentration of 3.5 μM (b2) also caused Sytox Green fluorescent dye to enter the cells, but the fluorescence was less clearly visible suggesting a lower degree of membrane perturbation. Quantification of the Sytox Green staining of the cells suggests an increase in membrane perturbation with an increase of concentration, as shown in the graph. Microscopy images are overlays of phase contrast and fluorescence images. Treatment was for 5 min and results represent three biological repeats. Scale bar of microscopy images represent 2 μm .

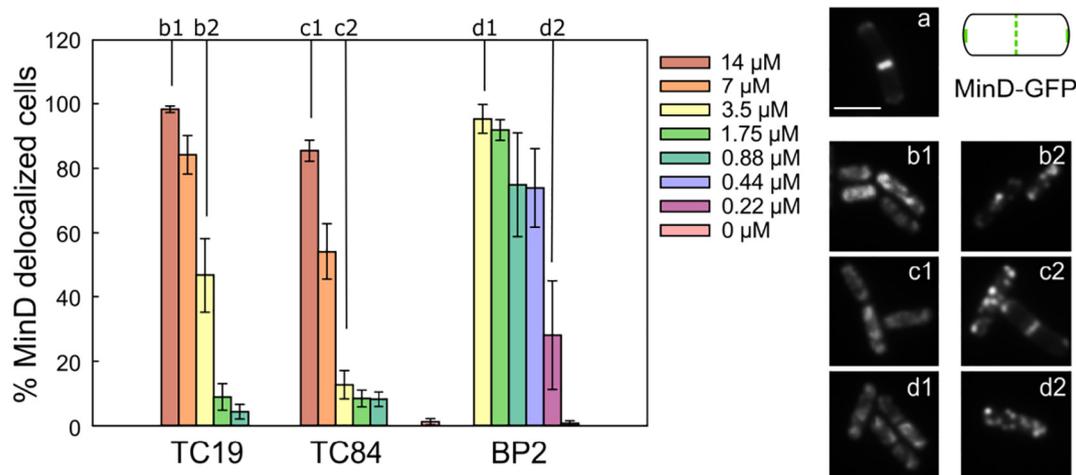


Fig. 7. Delocalization of membrane bound protein MinD after peptide treatment using *B. subtilis* mutant MinD-GFP. MinD-GFP is localized at the cell poles and septa as shown in the fluorescence microscopy image of the untreated *B. subtilis* mutant MinD-GFP (a). MinD-GFP delocalizes when the membrane potential is dissipated. Delocalization of the MinD-GFP was observed after treatment with 14 μM and 3.5 μM of TC19 (b1 and b2) and TC 84 (c1 and c2), and with 3.5 μM and 0.22 μM BP2 (d1 and d2). Treatment was for 5 min. The graph shows the % of cells with delocalized MinD-GFP from a total of about 600 cells analysed per treatment. The standard error bars represent three biological repeats. A total of 200 cells were counted of each biological repeat. Scale bar represent 2 μm .

delocalized. At lethal concentrations, 14 μM TC19 (b1) and TC84 (c1), and 3.5 μM BP2 (d1), MinD-GFP was evenly distributed throughout the cells. At sub-lethal concentrations, 3.5 μM TC19 and TC84, and 0.22 μM BP2, the appearance of MinD-GFP was “spotty”. Such a “spotty” appearance of MinD-GFP when delocalized has also been observed for *B. subtilis* treated with compounds that dissipate the membrane potential, valinomycin or CCCP [76]. However, the even distribution of the MinD-GFP as observed after exposure to the high TC19, TC84 and BP2 concentrations has never been reported. We suspect that at lethal peptide concentrations, MinD-GFP dissociates from the membrane due to rapid cell death, but at sub-lethal concentrations the protein remains attached but delocalized due to distortion of the membrane. Quantification of the microscopy images showed that MinD-GFP delocalization, both “spotty” and fully delocalized, occurred in a concentration dependent manner. This was the case for all GFP-fusion proteins observed. Therefore, only results for lethal concentrations are shown. We investigated our microarray data to observe whether *B. subtilis* is responding directly to the delocalization of MinD or its associated protein MinC, but we found no differential expression of MinD or MinC after 5 or 120 min of treatment with the peptides.

Delocalization or dissociation of the MinD-GFP occurred rapidly (≤ 5 min) (Fig. 7). This was also observed for MreB (Fig. 8). MreB is a cell shape determinant that forms an important part of the cell wall synthesis machinery and is pivotal in maintaining cell membrane homeostasis [77–79]. Both MinD and MreB requires the presence of a membrane potential for correct localization [76]. The transcriptomic analysis revealed that MreB was not differentially expressed after treatment with TC19, TC84 and BP2 (Table S7). Expression of the genes encoding the MreB homologous proteins Mbl and MreBH [78], however, were upregulated by 1.5 logFC and 4.6 logFC after TC19 treatment for 120 min (Table S7). After treatment with TC84 for 120 min MreBH were upregulated by 1.1 logFC. The upregulation of these genes suggests that *B. subtilis* respond to the delocalization of these membrane bound proteins.

The delocalization of MreB, together with the cell wall stress response, hinted at the possibility that other cell wall synthesis-associated proteins might also be delocalized. MreB forms a complex with MreC, MreD, RodA, MurG, MraY and several PBPs [80–82]. MurG was initially investigated as it has been shown to delocalize after treatment with the lipopeptide daptomycin, the cationic hexapeptide MP196, the cyclic decapeptide gramicidin S and the cyclic hexapeptide cWFW [34, 83, 84]. Treatment with TC19, TC84 and BP2 caused a rapid (≤ 5 min)

delocalization of the MurG-GFP. MurG is a *N*-acetylglucosamine transferase that catalysis the addition of *N*-acetylglucosamine to the *N*-acetylmuramic acid residue of lipid I, resulting in the formation of lipid II [51, 85]. The transcriptomic analysis revealed that *B. subtilis* responded to the treatment with TC19 after 120 min by upregulating MurG and genes co-transcribed with MurG, MurB, SpoVE, DivIB and Sbp (Table S7) [23, 86]. MurB was differentially expressed in response to TC84 after 120 min. In conclusion, it appears that *B. subtilis* adjust the expression of genes associated with MurG functionality in response to MurG delocalization.

Additional proteins involved in cell wall synthesis that delocalized after treatment with TC19, TC84 and BP2 were MraY-GFP, PBP2b-GFP, PonA-GFP and FtsW-GFP. Essential MraY is a phospho-*N*-acetylmuramoyl-pentapeptide transferase that catalyses the transfer of the phospho-MurNAc-pentapeptide moiety to undecaprenyl phosphate located at the membrane, forming lipid I [51, 85]. PonA is a class A penicillin binding protein (PBP) with both transglycosylase and transpeptidase activity [87–90]. MraY and PonA are both dependent on the proper localization of MreB [91, 92]. Penicillin-binding protein 2b (PBP2b) is a class B transpeptidase involved in the cell wall synthesis during cell division [90, 93]. PBP2b together with FtsW forms part of the divisome responsible for septal cell wall synthesis [94, 95] as well as for the stabilization of the Z-ring [94]. The Z-ring is formed by the polymerization of a tubulin-like protein, FtsZ, into a circular structure at mid-cell [96]. Delocalization of PBP2b, FtsW or FtsZ will prevent proper formation of the Z-ring, which serves as a scaffold for other proteins involved in synthesizing the septum or cell division site [49], and will thus interfere with cell division.

A general range of proteins involved in cell membrane synthesis (PgsA and PlsX), ATP synthesis (AtpA), Krebs' cycle and respiration (SdhA), cell division (FtsZ and DivIVA), transcription (RpoC), translation (RpsB) and DNA repair (DnaN and RecA) were selected to assess whether TC19, TC84 and BP2 affect other cellular functions due to membrane distortion. Delocalization of all proteins involved in cell membrane synthesis, ATP synthesis, Krebs' cycle and respiration, and cell division were observed. However, localization of RpoC and RpsB localized within the cytosol, and of DnaN and RecA, associated with the nucleoid, was not affected (Fig. 8). Clearly the abnormal localization of ATP synthase subunit AtpA is an indication of extensive perturbation of the cell membrane. In normal cells the AtpA complex is uniformly distributed at the membrane and its delocalization has been used previously as an indicator for abnormal membrane curvatures [77].

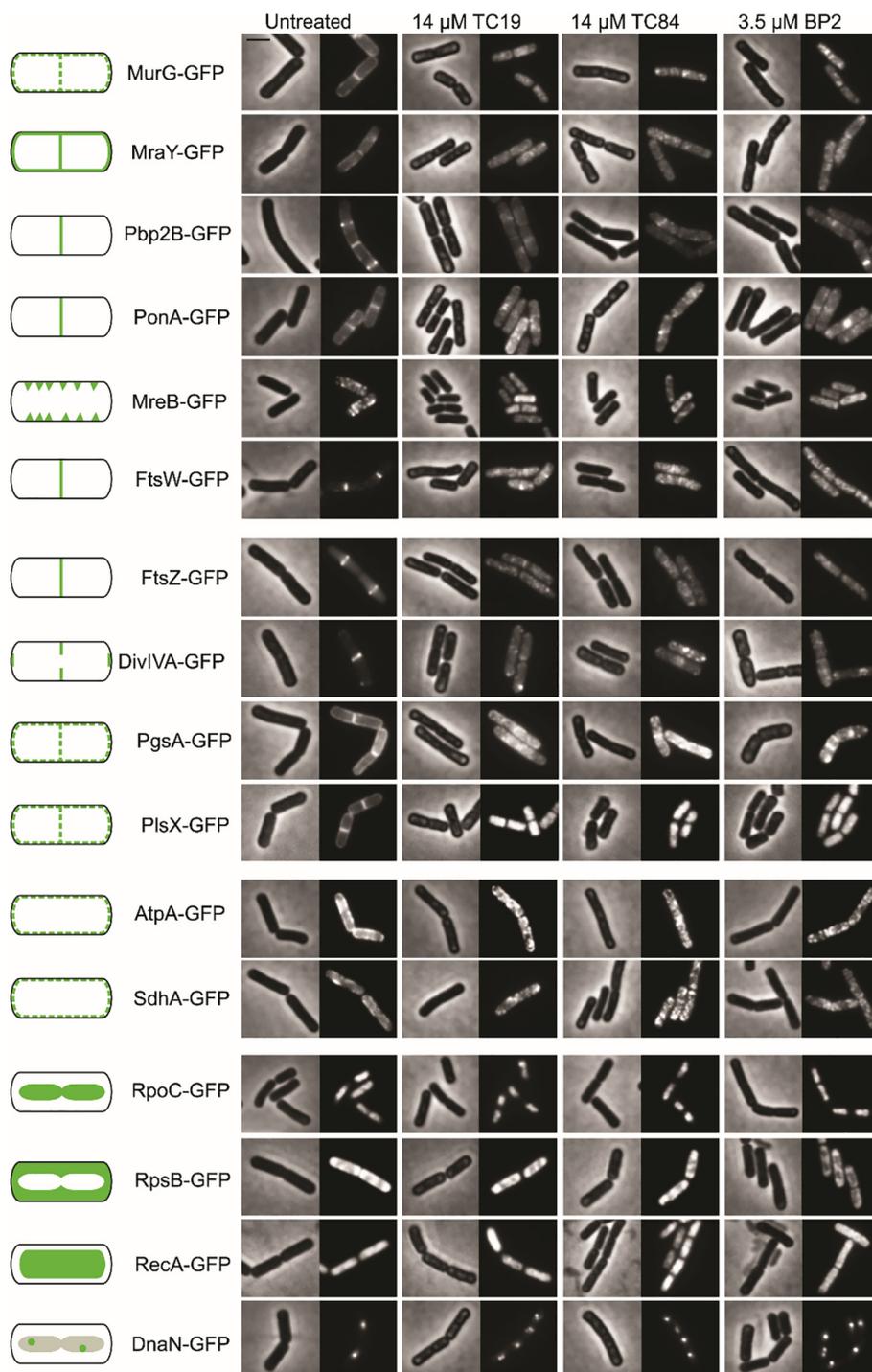


Fig. 8. Delocalization of proteins involved in various cellular processes after peptide treatment. *B. subtilis* mutants producing proteins fused to green fluorescent protein (GFP) were treated with lethal concentrations of TC19 (14 μ M), TC84 (14 μ M) and BP2 (3.5 μ M) for 5 min. The *B. subtilis* mutants expressed proteins involved in cell wall synthesis (MurG-GFP, MraY-GFP, PBP2b-GFP, PonA-GFP, MreB-GFP and FtsW-GFP), cell membrane synthesis (PgsA-GFP and PlsX-GFP), cell division (FtsZ-GFP, DivIVA-GFP), ATP synthesis (AtpA-GFP), Krebs' cycle and respiration (SdhA-GFP), transcription (RpoC-GFP), translation (RpsB-GFP) and DNA repair (DnaN-GFP and RecA-GFP). The comparison of images of treated and untreated cells showed that all proteins delocalized except proteins involved in transcription, translation, and DNA repair. Localization of proteins in untreated test conditions should not be over interpreted. Scale bar represent 2 μ m.

4. Discussion

TC19, TC84 and BP2 are predicted to share the common features of being cationic and amphipathic peptides and comply with the characteristics of their class by distorting the cell envelope of *B. subtilis* in a non-specific manner. TC19, TC84 and BP2 were rapidly bactericidal (≤ 5 min). Furthermore, as expected for this class of antimicrobial peptides, neither of these three peptides caused *B. subtilis* to develop resistance, as tested by 14 cycles of exposure to the peptides. TC19, TC84 and BP2 caused rapid membrane permeabilisation without causing overt cell lysis, as observed with the TEM and Sytox Green staining images. TC19, TC84 and BP2 at sub-lethal concentrations initiated a stress response distinctively different from known antimicrobials. We

compared the response with responses observed upon treatments that target the cell envelope by inhibiting cell wall synthesis (D-cycloserine, oxacillin, ristocetin, bacitracin, vancomycin and amoxicillin), distorting the cell membrane (i.e. the detergent Triton X-114), distorting the cell membrane without causing membrane permeabilisation (the ionophore monensin) and those that do permeabilise the membrane (gramicidin A and polymyxin B) [23]. An independent study using a proteomic approach also found that treating *Bacillus subtilis* with different membrane active compounds does not necessarily cause a similar stress response [97]. Our AMPs induced a stress response most similar to Triton X-114. Triton X-114 is a non-ionic detergent that causes a phase-separation and is often used to solubilize and separate proteins during extraction [98].

TC19, TC84 and BP2 induced a cell envelope stress response (CESR) by upregulating genes regulated by Sigma factors M, V, W and X and two component regulator systems (TCSs). *B. subtilis* responded to TC19 and TC84 similarly as when exposed to the natural cationic alpha-helical AMP LL-37 [99], by upregulating genes controlled by the LiaRS, YxdJK, and BceRS TCS regulators [26]. The YxdJK TCS has only been associated with cell membrane perturbation [26], but the LiaRS TCS have been linked with both cell wall synthesis inhibition and membrane perturbation [24–29]. The BceRS TCS and its cognate ABC transporters, BceAB, are upregulated in response to bacitracin and vancomycin, and are associated with cell wall synthesis inhibition [24, 30, 31]. Additionally, *B. subtilis* differentially upregulated the YtrA regulon in response to TC19 and TC84. The YtrA regulon has been associated with exposure to cell wall synthesis inhibiting compounds [24, 25, 32]. These findings suggest that TC19 and TC84 target the cell wall architecture and/or synthesis in addition to the cell membrane. However, LL-37 has to our knowledge not been shown to be involved in cell wall synthesis inhibition, and the upregulation of the BceRS TCS suggest that LL-37 is targeting cell wall synthesis. In the case of BP2, few genes were differentially expressed in response to the peptide. We speculate that it is due to the very rapid activity of BP2 that prevents the initiation of a stress response similar to what was observed for TC19 and TC84. The few genes that were upregulated suggested that BP2 might have a similar cell envelope target as TC19 and TC84. However, the physiological analysis showed that no cell wall damage or cell lysis occurred after 5 min of treatment with lethal concentrations of TC19, TC84 and BP2. Instead, the membrane distortion caused by these peptides did cause delocalization of proteins essential for cell wall synthesis, MurG, MraY, MreB, PonA, PBP2b and FtsW, within 5 min of treatment. Exposure to LL-37 also caused delocalization of these membrane bound proteins involved in cell wall synthesis (Fig. S5), suggesting that this delocalization may be a general effect of exposure to cationic amphipathic antimicrobial peptides acting on the membrane, since delocalization of membrane bound cell wall synthesis proteins has also been reported for non-pore-forming linear hexapeptide MP196, lipopeptide daptomycin and non-pore-forming cyclic hexapeptide cWFW [34, 83, 84]. The changes which TC19, TC84, BP2 and LL-37 cause to the membrane must be different to those caused by daptomycin, since daptomycin did not affect the localization of integral membrane proteins MraY and PBP2b [84]. The activity of TC19, TC84 and BP2 on the cell membrane also caused other proteins involved in cell membrane synthesis and metabolism to delocalize. Delocalization of such proteins prevents their normal functioning [76, 100] and will be deleterious for the survival of the cells. The delocalization of membrane proteins involved in cell wall synthesis by TC19, TC84, BP2 and LL-37 suggests that these amphipathic AMPs have common elements in their mode of action, some of which might be mediated through a shared secondary structure. LL-37 and BP2 are referred to as alpha-helical [9, 99] while we have preliminary indications that at least TC84 acquires some alpha-helical features upon interaction with membrane mimetics (unpublished observations).

5. Conclusion

TC19, TC84 and BP2 showed to be promising candidates as antimicrobial agents against Gram-positive bacterial cells as their membrane perturbation activity causes interference with various essential cellular processes leading to death. We observed no adaptation or resistance development against TC19, TC84 and BP2. No difference in mode of action between TC19 and TC84 was found, but BP2 was more rapidly active compared to TC19 and TC84 which we suspect is due to its higher cationic charge. We employed a transcriptomic approach to elucidate the mode of action and observed that the membrane perturbation caused by the cationic amphipathic peptides induced a membrane and cell wall stress response. With the aid of the green fluorescent protein fused to essential proteins bound to the membrane, we were

able to establish that proteins involved in cell wall synthesis are delocalized. Delocalization will have a deleterious effect on the normal functioning of the proteins. Conclusively, our findings have elucidated crucial aspects of the mode of action of TC19, TC84 and BP2. The observed lack of readily emerging resistance implies significant potential for further preclinical studies aimed at clinical development.

Transparency document

The <http://dx.doi.org/10.1016/j.bbamem.2018.06.005> associated with this article can be found, in online version.

Acknowledgements

The authors acknowledge Edward A. de Koning and Terrens N.V. Saaki for constructing the strains EKB40, and TNVS30D, respectively, and Urša Gubenšek for constructing the strain UG-10 during her Master's internship. We thank the MicroArray Department (MAD) of the University of Amsterdam for their contribution to the transcriptomic analysis. S. Ouardien acknowledges the Erasmus Mundus Action 2 program (EMA2) and University of Amsterdam for funding. M. Riool and S. A. J. Zaat are supported by the FP7-HEALTH-2011 grant 278890, BALI – Biofilm Alliance. The authors declare that they have no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbamem.2018.06.005>.

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