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Emerging approaches to study cell-cell interactions

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***In vitro* supramolecular modification of L-form bacteria using lipidated coiled-coil peptides**

5.1 Introduction

The biotechnology industry enjoys more success and influence than ever before.¹² Biotechnological innovations and discoveries help to improve the quality of life worldwide. Industrial biotechnology uses microorganisms to produce chemicals, materials and energy.³⁴ One of the most important producers are actinomycetes, which are best known for producing a plethora of useful compounds.^{5,6} Among them are about two-thirds of all antibiotics that are used in the clinic, as well as various antifungals, anthelmintics and anti-tumour agents.^{7,8} Despite this vast arsenal of useful metabolites, we are facing a massive challenge concerning the alarming increase in the number of bacteria that have become insensitive to virtually all antibiotics.⁹⁻¹¹ Therefore, there is a need to find new antimicrobials and new biotechnological production methods. For example, elimination of the cell wall could

increase the secretion of proteins and prevent the synthesis of cell-wall fragments which can have toxic immune-stimulatory effects.^{9,12,13} This has also spurred interest in finding new compounds in numerous ways, for instance by isolating compounds from untapped sources,^{8,14} or by cultivating different organisms together.¹⁵ The latter could induce activation of biosynthetic gene clusters that are otherwise inactive.¹⁶ Co-cultures could potentially also lead to novel chemical structures due to the combined biochemical activity of the organisms that grow together. However, finding suitable conditions for co-culturing can be problematic and time-consuming when more than two different organisms are involved. This severely limits its use on a large scale.

An attractive alternative to synthetic communities is to create synthetic hybrid cells, which contain chromosomes of different organisms equipped with a multitude of biosynthetic gene clusters. One factor that makes a fusion between cells complicated is the presence of the cell wall, which envelopes bacteria and protects them from harsh environmental conditions. In theory, protoplasts, which are generated by enzymatically degrading the cell wall, could be used. Indeed, protoplast fusion has been used in the past in classical strain crossing approaches but is limited to closely related species. Furthermore, protoplasts are intrinsically unstable and will immediately regenerate a cell wall, thereby effectively making them fusion-incompetent.¹⁷⁻¹⁹ A better alternative would be to use cells that can indefinitely propagate without their cell wall. This alternative dramatically increases the time and thus likelihood for fusion to take place. These variants, called L-forms (or L-phase, L-phase bacteria, or cell-wall-deficient bacteria (CWD)), have been generated from a broad range of bacteria, including the antibiotic-producing actinomycetes, by inhibiting crucial steps in the biosynthesis pathway of the cell wall (Figure 1).^{13,18,20} L-forms

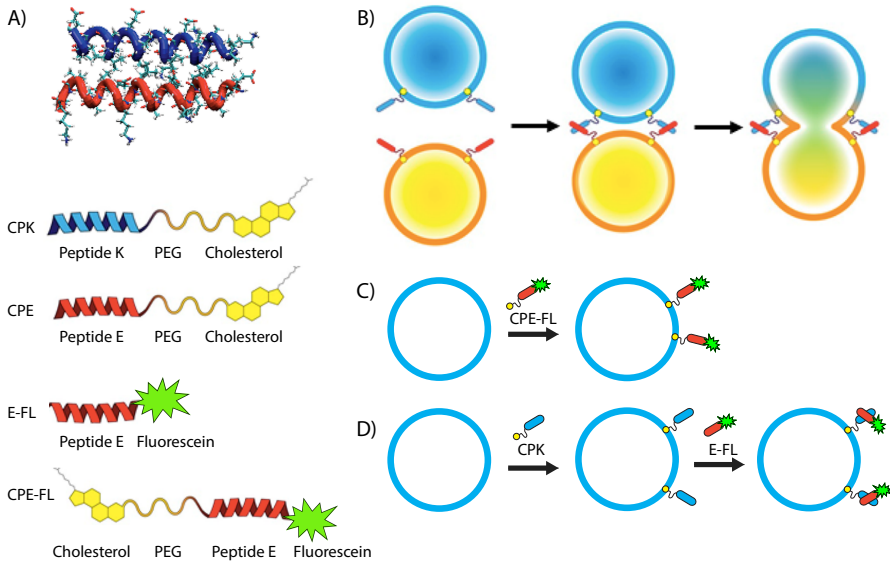
morphology is adaptable and allows cells to surpass environmental difficulties, for example, hyperosmotic stress conditions or treatment with antibiotics.²¹ The complete absence of the cell wall has critical physiological consequences: like protoplasts, L-form cells are highly sensitive to osmotic changes and require high levels of sucrose for osmoprotection.⁹ Additionally, the proliferation of such L-form cells no longer depends on the highly organised cell-division machinery, but is based on simple biophysical principles.^{7,17,22} Claessen *et al.* have expanded on this research by providing proof that cell-wall-deficient strains can propagate solely under hyperosmotic stress conditions.⁹ This evidence is a milestone for the environmental relevance of such bacteria.⁹ As a result, these cells have been suggested to resemble primordial life forms that existed well before the cell wall evolved.

Cell-cell fusion can be achieved by a variety of approaches, including chemical, biological and physical methods.²³ Membrane fusion is a controlled process in nature and shows a broad variety, from viral and eukaryotic to extracellular fusion, and is regulated by a variety of functional proteins.²⁴ Previously, fusion of plant protoplasts was induced by polyethylene glycol (PEG)⁹⁹ and since then it has been applied in many different applications including mammalian-cells fusion for the development of hybridomas, albeit with very low efficiency.²⁵ During neuronal exocytosis, transport vesicles are being docked to the plasma membrane by the formation of coiled-coils within complementary SNARE protein subunits, located on the cell membrane.^{24,26} Moreover, SNARE proteins have been associated with the formation of autophagosome and also have been identified between mammalian and fungus cells.²⁷ Additionally, SNARE proteins function in the processes of small shuttling vesicles like neuronal exocytosis, or bigger transport containers such as the proenzyme

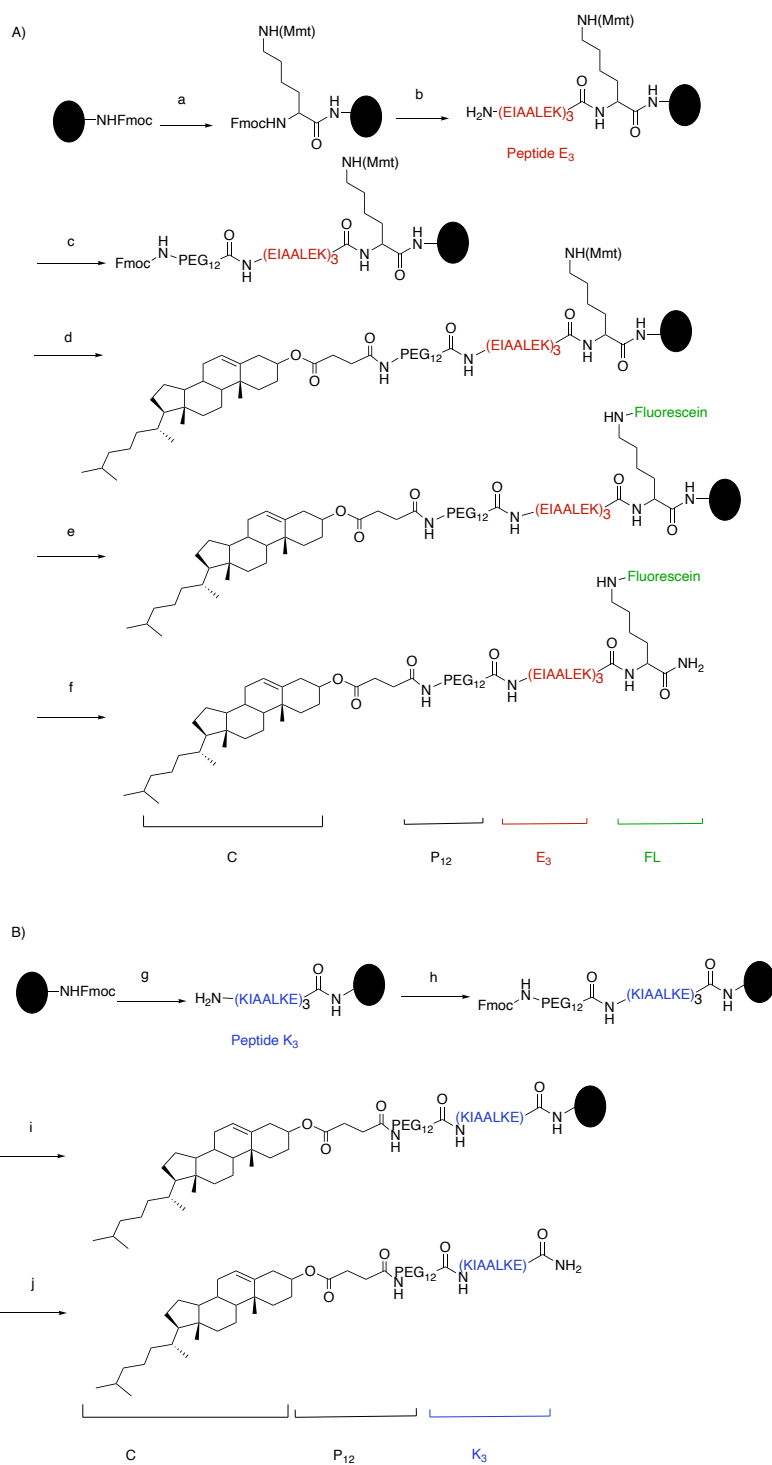
granules of pancreatic cells. In this process, complementary SNARE protein subunits present on opposing membranes assemble into an interacting structure by coiled-coil formation.²⁸⁻³⁴ As a result this assembly drives the facing cell membranes into close proximity, resulting eventually in lipid mixing and associated content transfer.

The application of supramolecular concepts *in vitro* and *in vivo* in order to study the impact in biological processes is relatively new. One approach includes chemically adjusted systems being inserted into cell membranes in order to modify or regulate cellular behaviours via external synthetic signals.³⁵ Lipidated peptides have been used extensively in biological systems because their behaviour can be carefully regulated by modifying the cargo's hydrophilicity and the anchor's hydrophobicity resulting in membrane incorporation.³⁶ However, typically, this procedure disrupts normal cell function of the resulting hybrid cells, and the number of surviving cells is very low.³⁷ To overcome this barrier, a simple and straightforward biomimetic model system inspired by the eukaryotic SNARE proteins was used. Many different synthetic systems have been designed in order to imitate membrane fusion. However, the essential characteristics of natural membrane fusion are not always achieved; for example coupled surface recognition, helical coiled-coil formation and disruption of the contact site which is followed by fusion; i.e. content and lipid mixing.³⁸⁻⁴⁰ An entirely artificial membrane fusion system which retains the essential characteristics of SNARE-mediated fusion has been designed by the Kros laboratory and consists of a pair of lipidated coiled-coil peptides (Scheme 1).^{28,41-44} In this system, the complementary lipid-peptide conjugates (CPK₃ and CPE₃) are comprised of the peptides K₃ [(Lys-Ile-Ala-Ala-Leu-Lys-Glu)₃] and E₃ [(Glu-Ile-Ala-Ala-Leu-Glu-Lys)₃], which are linked via a polyethylene

glycol (PEG) spacer to a cholesterol moiety (Scheme 2). As a result these lipidated peptides readily insert into the membrane bilayer due to the hydrophobic cholesterol anchor (Scheme 1).⁴⁵



Scheme 1 A peptide-mediated model system for membrane fusion. (A) The coiled-coil structure of peptides E and K (adapted from PDB 1UOI), the lipidated peptides CPE and CPK, fluorescently labelled peptide E-FL and the fluorescently labelled lipidated peptide CPE-FL. (B) Targeted L-form bacteria fusion mediated by coiled-coil formation between CPE modified L-forms and CPK modified L-forms. While the two populations are mixed (left), coiled-coils are formed between the two complementary peptides (middle), and cell fusion occurs (right). (C) Insertion of fluorescently labelled lipidated E peptide (CPE-FL) into plain alpha L-form bacteria membrane. (D) CPK inserts into plain alpha L-form bacteria membrane, and coiled-coil is formed with the fluorescently labelled peptide E. (Images are not to scale)



Scheme 2 Synthetic procedure of lipopeptide CPE-FL (A) and CPK (B). (A) Reagents and conditions: a) (1) Resin deprotection using 20% piperidine in DMF, rt, 30 mins; (2) Fmoc-Lys(mmt)-OH, HOBt, DIC, DMF, overnight, rt; b) solid phase peptide synthesis; c) Fmoc-PEG_n-COOH, HOBt, DIC, DMF, overnight, rt; d) (1) 20% piperidine in DMF, rt, 30 mins; (2) cholesteryl hemisuccinate, HOBt, DIC, DMF, overnight, rt; e) (1) 1% TFA/DCM, rt, 10 mins (3 times); (2) 5(6) Carboxyfluorescein, HOBt, DIC, DMF, overnight, rt; f) TFA/TIS/H₂O (95:2.5:2.5 v/v) for 1.5 h. (B) Reagents and conditions: g) solid phase peptide synthesis; h) Fmoc-PEG_n-COOH, HOBt, DIC, DMF, overnight, rt; i) (1) 20% piperidine in DMF, rt, 30 mins; (2) cholesteryl hemisuccinate, HOBt, DIC, DMF, overnight, rt; j) TFA/TIS/H₂O (95:2.5:2.5 v/v) for 1.5 h.

In this chapter a supramolecular approach was applied to induce coiled-coil formation at the surface of L-form bacterial membranes (Scheme 1). In turn this allows an extensive variety of molecules to be conjugated onto the L-form outer surface via noncovalent interactions. In the first step, the lipidated coiled-coil peptides CPE/CPK insert immediately into the cell membranes due to the cholesterol moiety via hydrophobic interactions while the PEG_n spacer provides the sufficient flexibility which is needed to facilitate potent molecular recognition between the two complementary coiled-coil peptides. For this, a solution of the cholesterol-modified peptide E or the cholesterol-modified peptide K was added to L-forms, resulting in the spontaneous integration of these peptides into the L-form membrane. In the second step, the complementary coiled-coil peptide was added resulting in coiled-coil formation.

This chapter presents evidence – for the first time – of surface modification of living L-form cells, as a first step towards to L-form fusion, using cell-compatible coiled-coil peptides. L-form modification resulting in fusion will enable the study of fundamental questions related to the origin of cellular complexity, regeneration of different actinomycetes, and strain improvements.

5.2 Results and discussion

Initially, testing was done to ascertain whether the L-form bacterial membrane incorporates cholesterol-modified coiled-coil peptides. In order to demonstrate coiled-coil formation at the surfaces of L-form bacteria, the ability of the cholesterol moiety to insert into the L-form membrane was investigated. Alpha L-forms (wild-type non-fluorescence L-forms (1000 cells/ μ L) derived from actinomycetes) were incubated for 30 minutes with carboxyfluorescein-labelled CPE (CPE-FL) at a final concentration of 5 μ M. Subsequently, confocal microscopy and flow cytometry were used to visualise and quantify CPE incorporation within the treated cells. As expected, L-forms became highly fluorescent, indicative of successful CPE-FL incorporation (Figures 1 and 2).

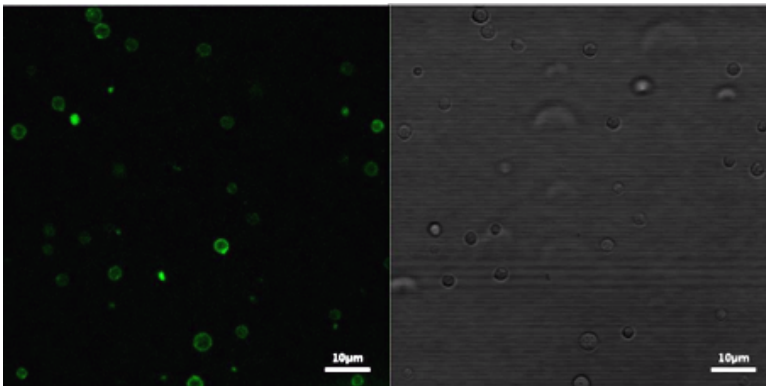


Figure 1 Confocal microscopy images (fluorescence, bright field) of alpha L-forms cells. The L-forms were treated with 5 μ M final concentration of fluorescently labelled CPE (CPE-FL) at 37 °C. After 30 mins confocal microscopy images were taken. Scale bar: 10 μ m.

An investigation was then undertaken into whether the peptides at the L-form surface were accessible for coiled-coil formation with complementary peptide K. For this, alpha L-forms were incubated with fluorescent coiled-coil peptides in the presence or absence of a cell membrane anchor. A 5 μ M CPK solution was added to L-forms for 30 minutes and unbound CPK was removed by washing the L-forms. The L-forms were then incubated for 10 minutes with a 10 μ M solution of carboxyfluorescein- labelled peptide E (FL-E). After three washing steps in order to remove any free FL-E, confocal microscopy and flow cytometry were used to visualise and quantify the efficiency of modification of the treated cells. As expected, the membrane of the L-forms was highly fluorescent, indicating that the CPK was successfully incorporated. When addition of CPK to L-forms was omitted, no fluorescence could be observed (Figure 2A). This confirmed successful coiled-coil formation between the CPK inserted into the L-form membrane and peptide FL-E. Furthermore, the reversed path also resulted in coiled-coil formation by first adding lipidated peptide CPE to the L-forms, followed by incubation with the fluorescently labelled peptide K (FL-K) (Figure 2A).

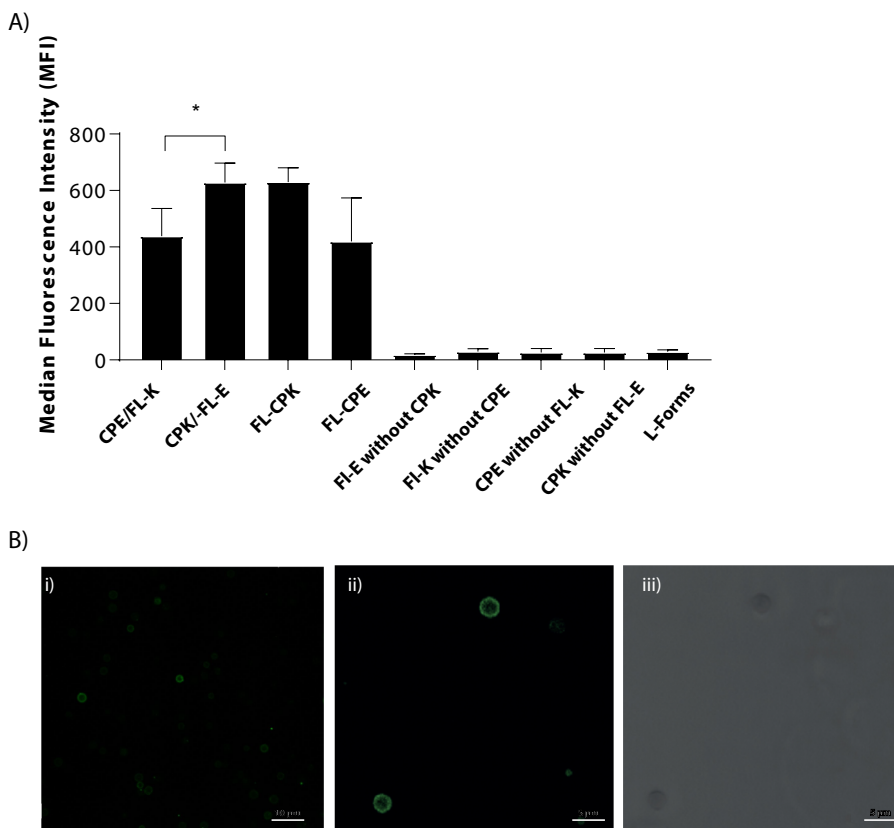


Figure 2 Alpha L-form bacteria incubated with different types of fluorescent peptides. (A) Flow cytometry indicates coiled-coil labelling of L-form membrane. (B) Confocal microscopy images of CPK/FL-E modified L-forms (i scale bar: 15 μ m; ii-iii scale bar: 5 μ m). The assay was set up in triplicate. Data are represented as median \pm SD. Error bars SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Thus, the membranes of cell-wall-deficient bacteria can be modified by this system without using complicated and time-consuming protocols such as protein engineering.⁴⁶⁻⁴⁸ Not only can the required coiled-coil peptides be synthesised within a day but this overall supramolecular approach for cell membrane modification is also fast. To date, the exact membrane composition of L-form bacteria is not known, and membrane biophysics could influence the lipid insertion. However, L-forms treated with either CPK-FL or CPK/FL-E showed around

1.5-fold higher fluorescence intensity than the respective L-forms which were treated with CPE-FL or CPE/FL-K (Figure 2A). This flow cytometry analysis seems to indicate that the membrane composition favours the positively charged peptide K over the negatively charged peptide E. Nevertheless, more studies are needed in order to clarify the individual behaviour of these coiled-coil peptides at the L-form surface.

These initial studies demonstrated the formation of coiled-coils at the surface of L-form bacteria by directly adding a cholesterol peptide-conjugate in the L-Form bacteria medium, followed by the incubation with the complementary peptide. This enables the docking of different molecules and nanoparticles on the surface of bacteria using this method. Kros *et al.* have shown that by the formation of coiled-coils between peptides E and K, 100 nm liposomes can be bound at surfaces of various materials.⁴⁹ Also, coiled-coils mediate docking of liposomes on mammalian cells *in vitro* as well as *in vivo* on the surface of one-day-old zebrafish embryos.^{29,41,45} For this reason, it was interesting to study whether liposome docking is also possible at a cell-wall-deficient bacterial membrane. For this, the membrane of L-form bacteria was functionalised with CPK, and next fluorescent liposomes containing 1 mol% SR-B (sulforhodamine B) and 1 mol% CPE were added for 5–10 minutes. Moreover, in the same set-up, in order to accelerate the liposome-fusion events polyethylene glycol 1000 (PEG1000) was used as an additional fusogenic agent. It has been reported that during PEG-induced cell fusion (in concentrations between 30-50% w/w PEG) polyethylene glycol brings cell membranes into closer distance via dehydration and redistribution of intramembrane particles (IMPs).⁵⁰ Viability and fluorescent assays performed showed that concentration higher than 10% w/w of PEG could influence membrane integrity

(Figures 4 and 5). Thus, L-form bacteria were either incubated in the presence or absence of 5% w/w PEG1000 for one hour at 37 °C before liposome addition. Excess PEG1000 was removed and the cells were washed three times before the flow cytometry assay (Figure 3).

In this chapter flow cytometry was used to characterize L-form fusion events and this technique is briefly introduced here. In flow cytometry, forward scattered light (FSC) is related to the size of the particles, side scattered light (SSC) to their complexity and granularity.⁵¹⁻⁵³ The small size of the L-forms could pose a limitation for flow cytometry examination, mainly because of the difficulty to distinguish between cellular debris and small cells. Both fluorescence and forward scatter were used as dual trigger signals in order to successfully deal with this problem.^{51,53} The properties measured include a particle's relative size (SSC-A vs. FSC-A), relative granularity (SSC-A vs. FSC-A) and relative fluorescence intensity. FSC presents an appropriate method of recognising particles greater than a given size without taking into consideration their fluorescence.⁵¹⁻⁵³

Flow cytometry data from the L-form/liposome-docking assay revealed that the L-form bacterial membranes became fluorescent (Figure 3). In a control experiment, CPE liposomes were added to non-treated L-forms (i.e. no CPK pre-incubation) and, as expected, no fluorescence was observed (Figure 3). This confirms that using a complementary coiled-coil pattern, liposomes can be docked efficiently at the membranes of L-form bacteria. However, it was difficult to distinguish docking from fusion.

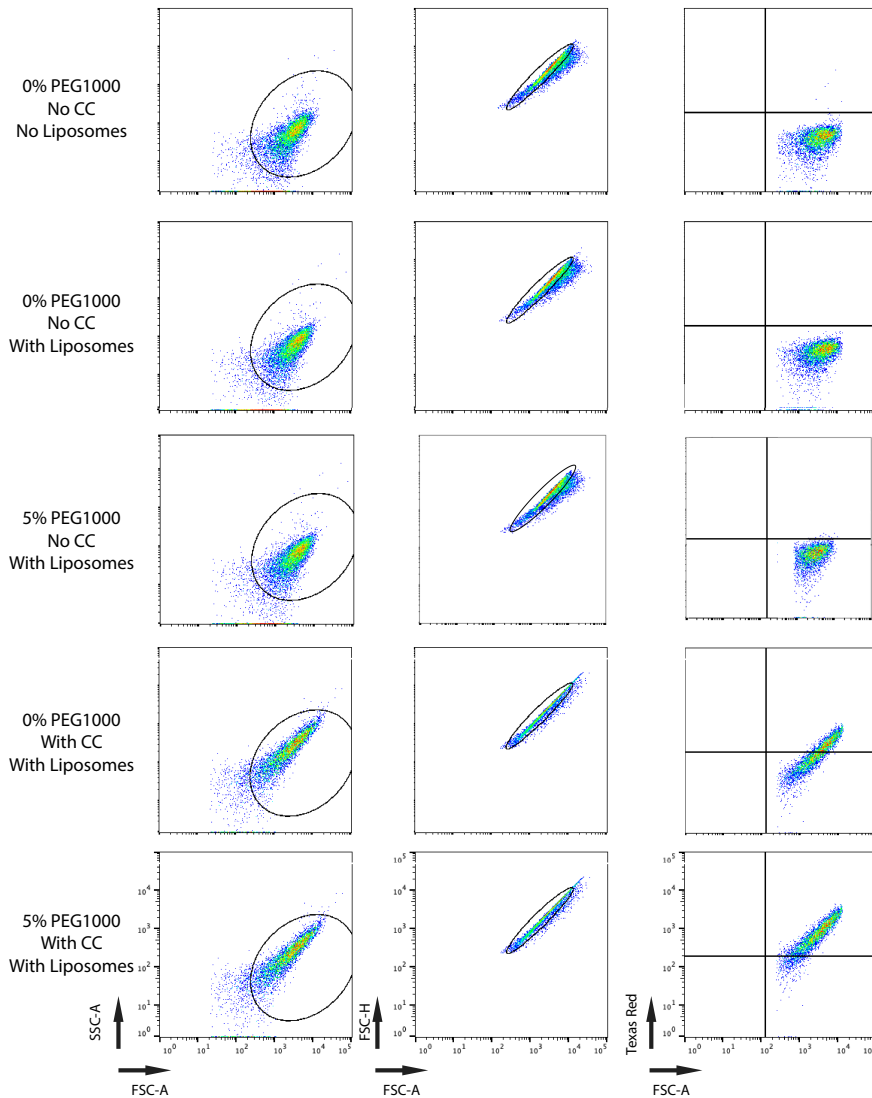


Figure 3 Flow cytometry shows liposomes docking at the surface of L-form bacteria. CPE-decorated liposomes (total lipid concentration 0.25 mM with 1 mol% lipidated peptide CPE) loaded with 20 mM sulphorhodamine (SR-B) were added to the L-forms, without prior addition of CPK peptides. L-forms were treated with 5 μ M final concentration of lipidated peptide CPK and were incubated for 5 mins. Subsequently, lipidated CPE-decorated liposomes (total lipid concentration 0.25 mM with 1 mol% lipidated peptide CPE) loaded with 20 mM sulphorhodamine (SR-B) were added to the L-forms. The L-form bacteria were incubated with or without 5% w/w PEG1000 for 1 h at 37 $^{\circ}$ C, before the excess PEG1000 was removed. All the experiments were performed at 37 $^{\circ}$ C.

Next, the viability of modified L-forms as well as the effect of polyethylene glycol 1000 (PEG1000) on L-form/L-form fusion was examined. For this, fluorescent derivative L-form strains constitutively expressing green fluorescent protein (eGFP) were created. The L-forms were incubated together with different concentrations of PEG1000 (0, 5, 10 % w/w) for one hour at 37 °C (Figure 4). Excess PEG1000 was removed and the L-forms were studied with confocal microscopy and flow cytometry measurements. This showed that 10% w/w PEG1000 disintegrates the L-form membrane resulting in loss of fluorescence (Figures 4 and 5A). In a separate experiment, L-form bacteria were treated with coiled-coil peptides and incubated together in the presence of different concentrations of PEG1000 (0, 5, 10 % w/w). L-forms were then plated on solid 1-phase medium (LPMA) and it was observed that some of the cells generated colonies, implying that these L-forms can proliferate (Figure 5). In addition, a live/dead viability assay was applied in order to visualise the viability of treated L-forms as an outcome of their membrane integrity. L-forms with an intact membrane will stain green (Cyto9, Figure 6), whereas dead or dying L-forms with a damaged membrane will stain red (PI). 10% w/w of PEG1000 dissociated the L-form membrane resulting in PI staining, whereas only Cyto9-labelled L-forms were observed when 5% w/w PEG1000 was used instead. In summary, this study showed that L-forms can proliferate after membrane modification.

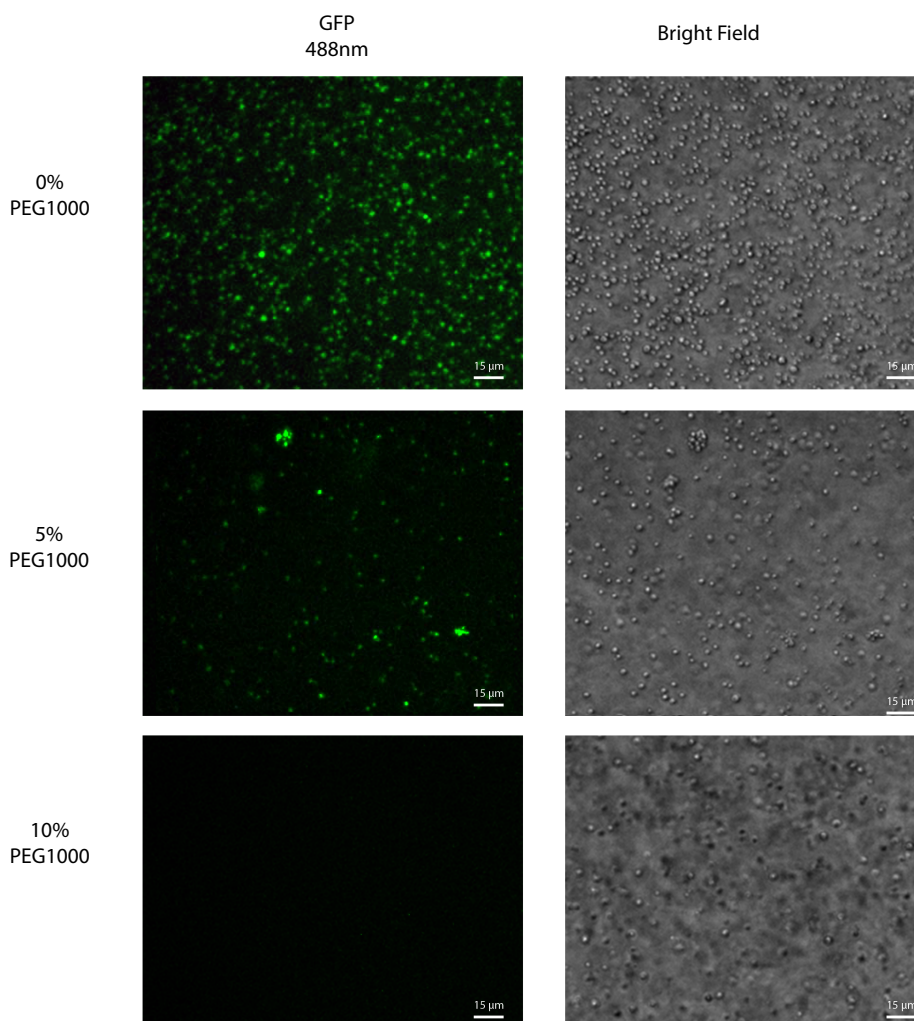


Figure 4 Confocal microscopy images indicate that a high concentration of PEG1000 is dissociating the L-form membrane. GFP expressing L-form bacteria were treated with different concentrations of PEG1000 (0, 5, 10 % w/w) for 1 h at 37 °C. The excess PEG1000 was removed before confocal microscopy. Scale bar: 15 μm.

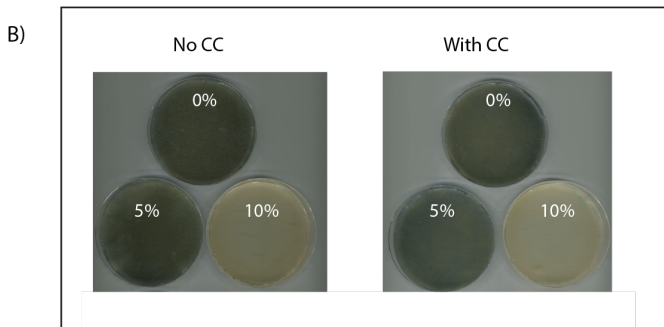
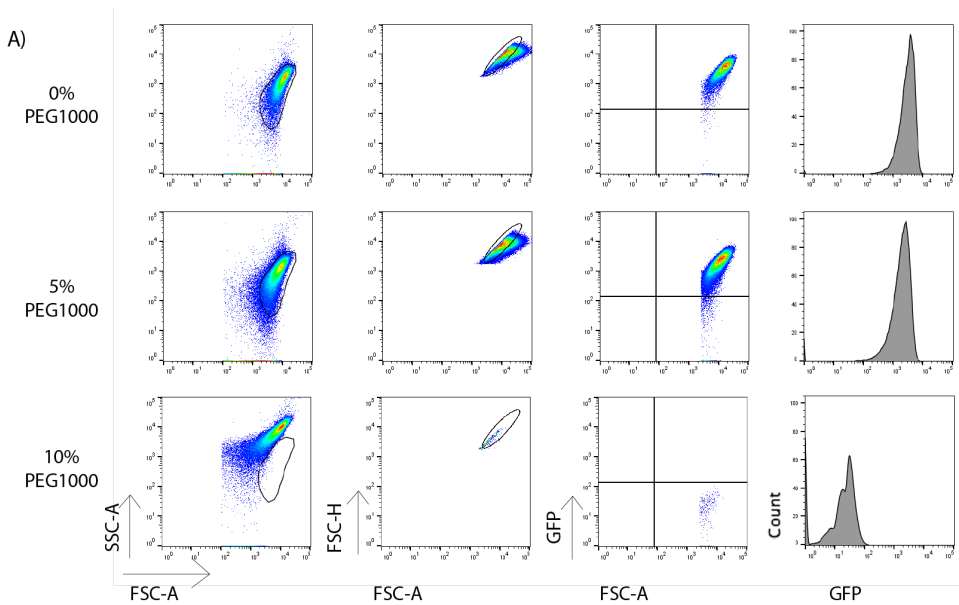


Figure 5 Flow cytometry and agar plates assay indicate that a high concentration of PEG1000 is dissociating the L-form membrane. (A) GFP expressing L-form bacteria treated with different concentrations of PEG1000 before confocal microscopy. (B) Agar plates with treated L-form bacteria showed the effect of PEG1000 on their viability. L-form bacteria were treated with coiled-coil peptides and were then incubated together with different concentrations of PEG1000 (0, 5, 10 % w/w) for 1 h at 37 °C. The excess PEG1000 was removed. L-form bacteria were plated on agar plates in order to observe colony formation.

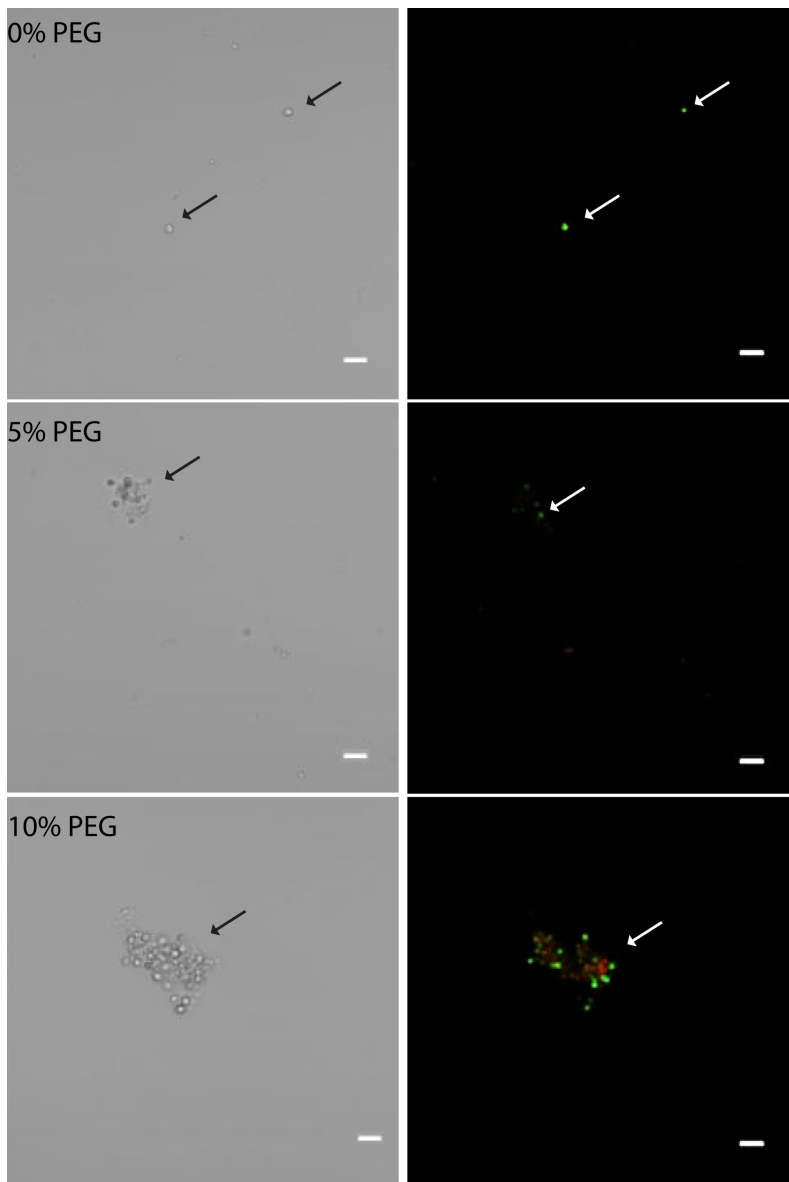


Figure 6 Confocal microscopy images indicate that a high concentration of PEG1000 is dissociating the L-form membrane. Viability labelling analysed with confocal microscopy using SYTO9/PI. Fluorescence images of the same samples at 528 nm for SYTO9 signal (green) and 645 nm for PI signal (red) are shown. L-form bacteria were labelled with PI and SYTO9. L-form bacteria were treated with coiled-coil peptides and were then incubated together with different concentrations of PEG1000 (0, 5, 10 % w/w) for 1 h at 37 °C. The excess of PEG1000 was removed. Dead L-form bacteria exhibit yellow or red fluorescence. Arrows point L-form bacteria. Scale bar: 10 μ m.

Next, a study was undertaken to ascertain whether the lipopeptides CPE/CPK could induce L-form fusion using flow cytometry in combination with confocal microscopy, as PEG-induced L-form fusion is inefficient. Genetically modified L-form strains constitutively expressing either mCherry or eGFP were created for this experiment. Next, mCherry- and GFP-expressing L-forms were incubated with 10 μ M of the lipidated coiled-coil peptides CPK and CPE respectively for 30 minutes, and any unbound lipopeptide was removed by washing the L-forms with media. In order to minimise L-form disruption during the washing steps, the cultures were collected and centrifuged at 2000 g for five minutes. Afterwards, the supernatant was carefully removed by decantation to avoid disturbance of the cells, and the cells resuspended in fresh LPB.⁹ These mCherry and GFP L-forms were mixed and incubated with concentrations of PEG1000 (0 or 5 % w/w) for one hour at 37 °C. Excess of PEG1000 was removed by centrifugation twice and replaced with fresh LPB media. Wojcieszyn *et al.* observed that fusion event occurs at PEG concentrations around 50% w/w.⁵⁰ Therefore, as a positive control, L-forms were incubated with 50% w/w PEG1000 (Figure 7). In this experiment, L-form aggregation was observed by confocal microscopy and flow cytometry. Unfortunately, fused L-forms were not observed.

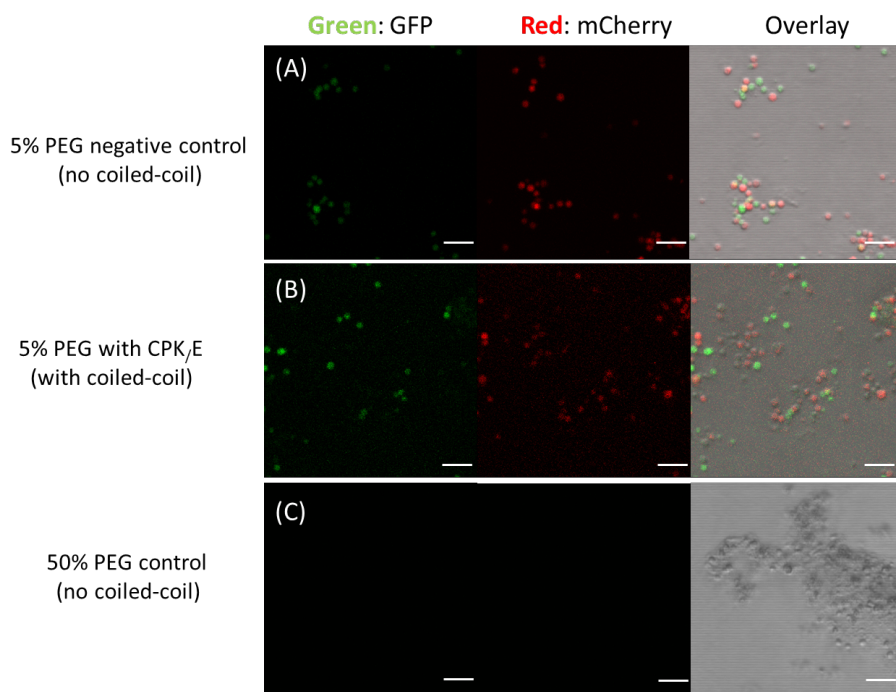


Figure 7 Cell-cell fusion using CPE/CPK-mediated coil-coil formation combined with 5% PEG1000. L-forms were incubated in the presence of 5% PEG1000 without lipopeptides (A) or with 10 μ M lipopeptides (B). GFP expressed L-forms were decorated with CPE and, upon mixing, mCherry expressed L-forms were decorated with CPK. Scale bar: 10 μ m.

Based on the light scattering characteristics, different populations of L-form bacteria were detected in the flow cytometry experiment: unfused/singlets, doublets/aggregates or fused cells. Singlets or aggregates of L-forms can be distinguished by their intrinsic characteristics. Flow cytometry results showed that there is a distinction between the effects of PEG1000 and coiled-coil modification (Figure 8). However, it was challenging to distinguish between fused with aggregated L-forms. It was assumed that PEG1000 induces aggregation and/or fusion of L-form bacteria, while the addition of lipidated coiled-coil peptides enhance the fusion (or aggregation) efficiency 3-fold. However, more control experiments are

required to confirm that L-form fusion occurred. For example, the effect of different salt concentrations or higher concentrations of coiled-coil peptides, with different PEG spacers and/or lipid moieties could have a more significant effect on L-form fusion. Moreover, as the molecular weight of polyethylene glycol (PEG) has a different effect on membranes⁵⁴, PEG with different molecular weights; (i.e. 400 Da, 6000 Da and/or 20000 Da) could be used as polymeric additives. Alternatively, antibiotic-resistant L-forms could be created and a dual-antibiotic selection applied to screen for fused L-forms.

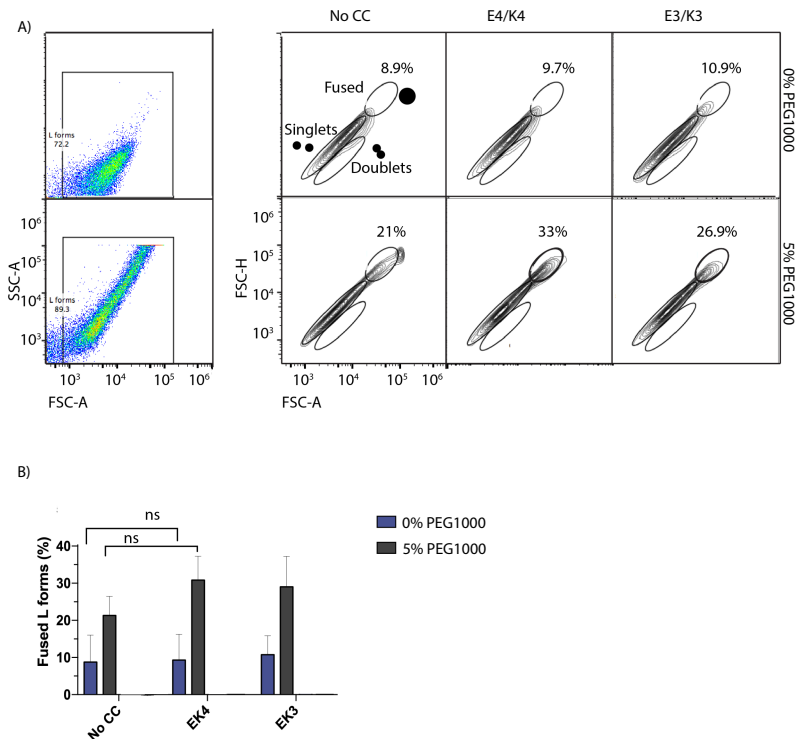


Figure 8 Quantification of L-form fusion using flow cytometry. (A) Dot and contour plots of windows and gating strategy used for the identification of unfused singlets or doublets of L-forms present in the L-form population. The percentages indicate the relative amount of gated L-forms (Singlets, Doublets, Fused). (B) Plots of flow cytometry analyses showing the number of L-forms from the tail-end of FSC(H)-FSC(A) plots (potentially fused) depending on the concentration of PEG1000 and different coiled-coil peptides. The assay was set up in triplicate. Data are representing as percentage \pm SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

5.3 Conclusions

A tool to modify the surface of L-form bacteria quickly and effectively was developed. A supramolecular method was used, with a complementary coiled-coil-forming peptide pair, in order to functionalise the membranes of L-form bacteria. Liposome docking tests proved that this approach can also be used to bind a wide variety of materials/ molecules on the L-form membrane. This generic method demonstrates that living L-form bacteria can efficiently be aggregated using a combination of SNARE protein mimics and PEG1000. It is expected that PEG1000 may promote the initiation of the fusion event by membrane dehydration, after which the coiled-coil peptides could facilitate the actual membrane fusion event. Because of the small size of the bacteria as well as light scattering limitations, it was difficult to distinguish aggregated from fused cells. To overcome these limitations in this experimental set-up, a double antibiotic selection assay could be designed to select for fused L-forms. Additionally, coiled-coil peptides with different PEG-spacers, different peptide length or lipid moieties might have a positive effect in L-form bacterial fusion. It is expected that this method will be able to induce fusion between L-form cells of distinct species, whereby the chemistry of two living cells is merged into a new cell. Successful fusion and regeneration of different actinomycetes strains at high frequency would facilitate the discovery of new antibiotics and microbial strain improvement.

5.4 Experimental methods

Method and materials

Rink amide resin, Fmoc-protected amino acids, and 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU) were purchased from Novabiochem. Trifluoroacetic acid (TFA), piperidine, diisopropylethylamine (DIPEA), N-methyl pyrrolidine (NMP), acetic anhydride, acetonitrile and dimethylformamide (DMF) were obtained from Biosolve. Triisopropylsilane (TIS), dichloromethane (DCM), diethyl ether, trimethylamine (TEA), cholesterol, succinic anhydride and sulforhodamine B were obtained from Sigma-Aldrich. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-glycerol-3-phosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids. Cholesteryl hemisuccinate and 5,6-Carboxyfluorescein were obtained from Sigma-Aldrich. The Fmoc-Lys(MMT)-OH was purchased from VWR. The Fmoc-NH-(PEG)_n-COOH spacers were purchased from Iris Biotech. PBS buffer contains 15 mM KH₂PO₄, 150 mM NaCl, 5 mM KH₂PO₄, and has pH 7.4. The polyethylene glycol 1000 (PEG1000) was purchased from Merck Millipore.

Preparation of L-forms

Bacterial strains (actinomycete *Kitasatospora viridifaciens*) used in this study were obtained from the Claessen Lab.⁹ To support growth of cell-wall-deficient (CWD) cells, strains were grown on solid medium L-phase medium (LPMA), containing 20% sucrose, 0.5% glucose, 0.5% peptone, 0.01% MgSO₄·7H₂O, 0.5% yeast extract, 0.75% Iberian agar (all w/v). After autoclaving, the medium was supplemented with MgCl₂ (final concentration of 25 mM) and 5% (v/v) horse serum. L-phase broth (LPB) was used as a liquid medium to support the growth

of wall-deficient cells. LPB contains 0.25% bacto-peptone, 0.15% yeast extract, 0.15% oxoid malt extract, 0.64 M sucrose, 0.5% glucose, 1.5% oxoid tryptic soy broth powder (all w/v) and 25 mM MgCl₂. Cultures were incubated at 30 °C while shaking at 100 rpm.⁹

1*10⁶ L-forms/ml with mCherry (red-fluorescent protein) expressed were incubated with 10 μM CPK, and 1*10⁶ L-forms/ml with GFP (green-fluorescent protein) expressed were incubated with 10 μM CPE for 30 minutes, then modified L-forms were washed with medium twice for, followed by the addition of 50% w/w PEG1000 stock medium, and medium results in L-forms incubated with different concentrations of PEG1000 (0%, 5%, 10% w/w).

Confocal microscopy imaging

L-forms were seeded in an 8-well slide (μ-Slide 8 well; Ibidi, Munich, Germany) at a density of 1*10⁵ L-forms per well in LPB. The fluorescent images were obtained using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) and analysed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The wavelength settings for GFP and mCherry were Ex/Em: 488/510 nm (Ex laser: 488 nm) and 585/610 nm (Ex laser: 532 nm), respectively.

Flow cytometry²¹⁻⁵³

L-forms were treated with 10 μM CPE/CPK peptides for 30 minutes, followed by two washing steps. In each washing step L-form cultures were collected and centrifuged at 2000 g for 5 minutes, before the supernatant was carefully removed by decantation and cells resuspended in fresh LPB. Then lipid-modified L-forms were incubated with different concentrations of PEG1000 (0%, 5%, 10% w/w). CPE-modified L-forms and CPK-modified L-forms were mixed and co-cultured for one hour at 37 °C. Flow cytometry assays were

performed using the Merck Guava® easyCyte 12HT Benchtop Flow Cytometer and all flow cytometry data were analysed using FlowJo™ v10.1 (FlowJo, LLC). Counting and characterization were performed by measuring 30,000 events in triplicate and concatenation of this data. For manual gating, the furthest ring of the dot plot was selected. Quadrants were selected manually to depict fluorescence plots. Compensation was not required.⁵¹⁻⁵³

Any granular material inside the cell, cell shape and surface, as well as the nucleus are the main factors that can affect light scattering.⁵¹⁻⁵³ A heterogeneous cell population was differentiated using correlated measurements of side-scattered light (SSC) and forward-scattered light (FSC). The former is comparable to cell internal complexity or granularity, whereas the latter is comparable to the cell-surface area or size.⁵¹⁻⁵³

Peptide synthesis

The cholesterol-modified peptides were synthesised as described elsewhere.⁴¹ An automatic CEM peptide synthesiser on a scale of 250 µM was used for the synthesis of peptides K, (KIAALKE)₃ and E, (EIAALEK)₃. For the synthesis, Fmoc chemistry was applied, and rink amide resin with a loading of 0.69 mmol/g was used. Amino acid couplings were performed with 4 eq of the appropriate amino acid, 4 eq of the activator HCTU and 8 eq of the base DIPEA. Fmoc deprotection was performed with piperidine: DMF (4:6 v/v). Synthesis of fluorescently labelled peptides was performed on rink amide resin. After initial Fmoc deprotection using 20% piperidine in DMF, Fmoc-Lys (MMT)-COOH was coupled, using 4 eq of DIC and 4 eq of HOBt, for one hour. The resin was used to synthesise acetylated peptide E and K along with CPE using standard Fmoc chemistry

procedures described earlier.^{28,41,44,55} Next, the side chain Lys(MMT) group was deprotected using TFA / DCM (1%) for 10 minutes (3x). The resin was then neutralised using DIPEA / NMP (10:90 v / v) for 10 minutes (3x). 5(6)-Carboxyfluorescein (Sigma-Aldrich) was coupled using 4 eq of DIC, and 4 eq of HOBt dissolved in DMF and coupling was performed overnight. Fluorescently labelled lipopeptide was cleaved from the resin by shaking the resin with a mixture of TFA / TIS / H₂O (95:2.5:2.5 v / v) for 1.5 hours. The cleavage mixture was precipitated in cold diethyl ether. The resulting fluorescently labelled crude products were purified by RP-HPLC using a Vydac C4 reversed phase column (214TP1022, 22 mm diameter, 250 mm length, 10 μ m particle size). With a linear gradient from 20% to 80% of acetonitrile in water with 0.1% TFA (v / v) over 36 minutes with the flow of 20 mL / mins, the crude lipopeptides were eluted. Sample elution was detected by UV detection at 214 nm and 256 nm. By using LC-MS (Gemini 3 μ C18 column coupled with Finningan LCQ advantage max (Thermo) ESI-MS analyse), the purity of the collected fractions was verified.

CP₁₂K₃: LC-MS m/z Calcd. [1673.6, M+2H]²⁺, found 1674.0. [1116.1, (M+3H)³⁺], found 1116.5. CP₁₂E₃: LC-MS m/z Calcd. [1675.0, M+2H]²⁺, found 1675.4. [1117.0, (M+3H)³⁺], found 1117.4. Ac-(EIAALEK)₃K-Carboxyfluorescein (FL-E): LC-MS m/z Calcd. [1405.2, M+2H]²⁺, Found 1405.6. [937.2, (M+3H)³⁺], found 937.6. Ac-(KIAALKE)₃K-Carboxyfluorescein (FL-K): LC-MS m/z Calcd. [1403.8, M+2H]²⁺, Found 1404.2. [936.2, (M+3H)³⁺], found 936.6. Cholesterol-PEG12-(EIAALEK)₃K-carboxyfluorescein (CPE-FL): LC-MS m/z Calcd. [1918.1, M+2H]²⁺, Found 1918.5. [1279.1, (M+3H)³⁺], found 1279.5. Cholesterol-PEG₁₂-(KIAALKE)₃K-carboxyfluorescein (CPK-FL): LC-MS m/z Calcd. [1916.7, M+2H]²⁺, Found 1917.1. [1278.1, (M+3H)³⁺], found 1278.5.

Liposome sample preparation

Stock solutions of lipids with total concentration of 1 mM in CHCl₃ (DOPE/DOPC/CH, molar ratios 2:1:1) and 50 μM peptide (molar ratio 1:1 MeOH/ CHCl₃) were prepared. The peptide solutions were prepared by taking the appropriate amount of peptide stock, evaporating the solvent over a stream of air, adding Hanks' Balanced Salt solution (HBSS) followed by sonication for one minute at 55 °C. The liposomes were prepared by using appropriate amounts of lipid and peptide stock solutions, evaporating the solvent over a stream of air, adding HBSS and sonication for one minute at 55 °C.

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