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Coiled-coil biomaterials for biological applications

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Chapter 5

L-forms Fusion Triggered by Coiled-coil Peptides



ABSTRACT

Genetic recombination plays an essential role in biological evolution. It not only increases biodiversity in nature but also helps species to survive in changing environments. Recombination by cell transfection or bacterial transformation is widely used in laboratory settings. However, gene recombination on a large scale remains challenging. Herein, we report fusion between cell wall deficient bacteria (*i.e.* L-forms) triggered by coiled-coil peptides, resulting in chromosome recombination. Two L-form strains with different antibiotic resistance and fluorescence markers were designed for this study. L-form cell-cell membrane fusion was obtained using the PK₄/CPE₄ coiled-coil peptide pair and the fused L-forms were enriched by double antibiotic selection in the liquid phase. The resulting L-forms were found to contain both antibiotic resistance and fluorescence markers. In addition, we studied the viability and cell division of enriched L-forms and chromosome segregation during the fused L-form division process on solid phase medium.

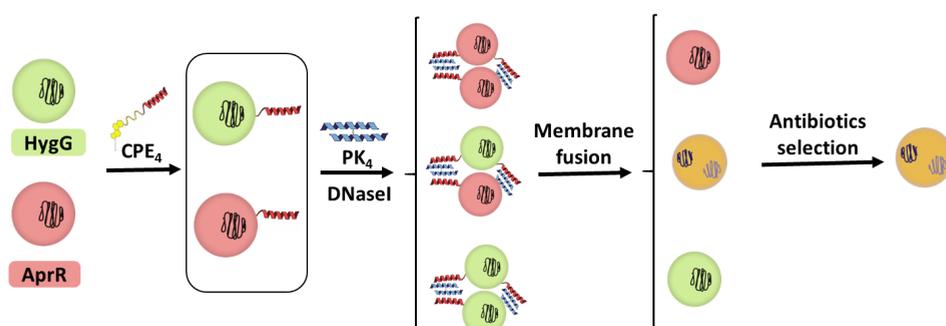
INTRODUCTION

The bacterial cell is a complex structure consisting of many components that each provide a function and purpose. A major component is the cell wall which provides shape, protection and resistance.¹⁻² It is however possible for a cell to survive without this cell wall, as seen for naturally wall-deficient cells of the *Mycoplasma* species and also in the case of stress-induced wall deficient L-forms.³ The application of stressors like osmotic stress, heat, pH, antibiotics and cell wall targeting enzymes can result in the formation of wall-deficient forms (S cells, L-forms, protoplasts), which ultimately reverse back to bacteria with a cell wall.⁴ These cells are often spherical in shape and either reproduce indefinitely in a wall less state (L-forms) or they are simply a transition phase (S cells or protoplasts).⁵ L-forms have been found to play a role in intracellular pathogenesis (*Listeria monocytogenes*, *Mycobacterium tuberculosis*),⁶⁻⁷ persisting infections (*Escherichia coli*),⁸ and food infections (*L. monocytogenes*) indicating the importance of studying their growth and function from an ecological perspective. Furthermore, the recent discovery of L forms derived from *Streptomyces* has triggered interest in their growth, antibiotic production and application.⁹

Streptomyces are a well-known resource of bioactive molecules, many of which are of commercial importance.¹⁰⁻¹¹ Apart from naturally produced antibiotics, the process of protoplast fusion has been applied to these species for genome shuffling to obtain innovative antimicrobials. The process involves generating protoplasts of the same or different species followed by fusion.¹² Indeed, this approach has yielded highly productive strains and novel antibiotics. However, a disadvantage is that the fused protoplasts revert back to the walled state, thus the recombined chromosomes would separate from each other. It was recently hypothesized that the use of L-forms, instead of protoplasts, for fusion could overcome this disadvantage and provide a framework for novel compound production.⁹ As a first step towards testing this hypothesis, we present in this chapter the first example of fusion between L-forms of the species *Kitasatospora viridifaciens*. This species is a tetracycline producer,¹³ therefore the fusion of such L-forms can be used to test the production of this antibiotic in future.

Coiled coils are fundamental folding motifs common in proteins and play an essential role in various processes of life.¹⁴⁻¹⁶ A well-known example of a functional coiled coil is the SNARE protein complex, which mediates membrane fusion.¹⁷⁻¹⁸ In our group, a synthetic membrane fusion model system based on

designed coiled-coil peptides was developed.¹⁹⁻²² This system is able to induce liposome-liposome and cell-liposome membrane fusion. In **Chapter 4** we presented a membrane fusion system based on the novel dimeric peptide PK₄ and its complementary lipopeptide partner CPE₄. This system is more fusogenic than our previously employed CPK₄/CPE₄ coiled-coil pair. In this chapter, we studied L-form membrane fusion initiated by the coiled-coil interaction between PK₄ and E₄ (Scheme 1). For the first time, we have achieved fusion of wall deficient L-forms of *Kitasatospora viridifaciens*. Combining fusion with antibiotic enrichment resulted in a cell culture containing more than 97% of the fused L-forms. We also investigated how and if chromosome segregation takes place after fusion. Through this work, we developed a feasible method to achieve L-form membrane fusion, which may have potential applications in studying genome shuffling and the development of new hybrid bacterial species.



Scheme 1. Coiled-coil peptides associate with L-form membranes and induce cell-cell fusion.

RESULTS AND DISCUSSION

1. L-form phenotype

In this study, permanent wall-deficient cells (L-forms) of *Kitasatospora viridifaciens* were prepared by exposure to lysozyme and penicillin G (a cell wall targeting antibiotic).⁵ In order to detect L-form fusion and select for the fused L-forms, two kinds of markers were introduced. A fluorescence marker (GFP or mCherry) was introduced for visualizing and tracking the cells during fusion with confocal microscopy and for quantification with flow cytometry. The introduction of antibiotic resistance markers (apramycin or hygromycin) enables selection and enrichment of fused cells. Two genetically modified L-form strains were obtained by transforming the wild type with the appropriate plasmids. The resulting HygG

strain expresses eGFP and is resistant to hygromycin B while the AprR strain expresses mCherry and has resistance to apramycin.

We first confirmed antibiotic resistance by determining the minimum inhibitory concentration (MIC) of each strain to both antibiotics (Figure 1A). The colony forming units (CFU) were used to quantify the minimum concentration of antibiotics required for killing each strain. The strain expressing resistance to apramycin (AprR) grew on all concentrations of apramycin while showing a MIC of 2 $\mu\text{g}/\text{mL}$ for hygromycin B. The strain expressing hygromycin resistance (HygG) grew on all concentrations of hygromycin B while showing a MIC of 20 $\mu\text{g}/\text{mL}$ for apramycin. These findings reaffirmed that the inserted resistance genes do not provide cross-resistance to the antibiotics used. Confirmation of the fluorescence reporters was obtained using microscopy with cytoplasmic mCherry detected in the AprR strain and GFP detected in the HygG strain (Figure 1B), no bleed-through was observed in the two channels.

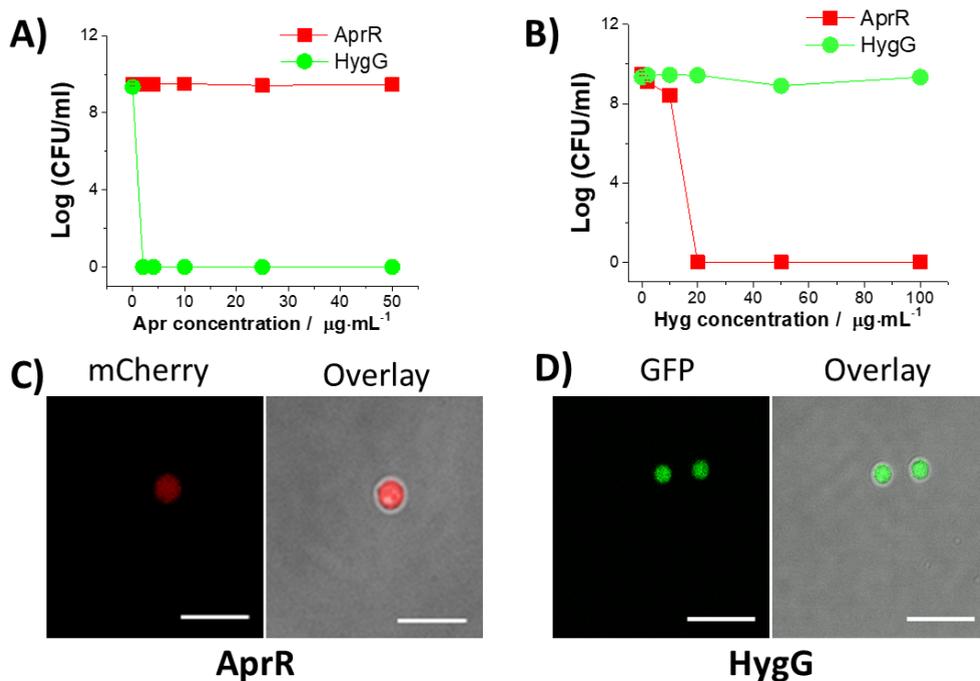


Figure 1. Antibiotic susceptibility testing: AprR and HygG strains grow in the presence of apramycin, A) or hygromycin, B). Fluorescence marker testing by fluorescence microscopy indicates a positive signal in the red channel for AprR, C) and in the green channel for HygG, D) Scale bar: 10 μm .

2. L-form membrane fluorescence labelling using coiled-coils

In order to apply the PK₄/CPE₄ coiled-coil membrane fusion system to trigger L-form fusion, it was necessary to first confirm that lipopeptide CPE₄ can be incorporated into L-form membranes. In previous studies, it was shown that the lipopeptide CPE₄ spontaneously incorporates into mammalian cell membranes.²³⁻²⁴ However the lipid composition of L-forms differs from mammalian cells, which could influence the efficiency of membrane modification. To investigate CPE₄ incorporation into L-form membranes, a fluorescence labelling assay was performed (Figure 2A). Wild type L-forms devoid of any fluorescence were used in this assay. As expected, in the absence of CPE₄, fluorescein-labelled peptide K₄ (fluo-K₄) did not bind to the L-form membrane (Figure 2B). With CPE₄ present in the membrane, fluo-K₄ did bind to the lipopeptides resulting in homogenous labelling (Figure 2C). These results confirm that the cholesterol anchor inserts into the L-form lipid membrane and thus modification of the membrane with CPE₄ is possible. In addition, it can still form coiled coils with the complementary peptide K₄.

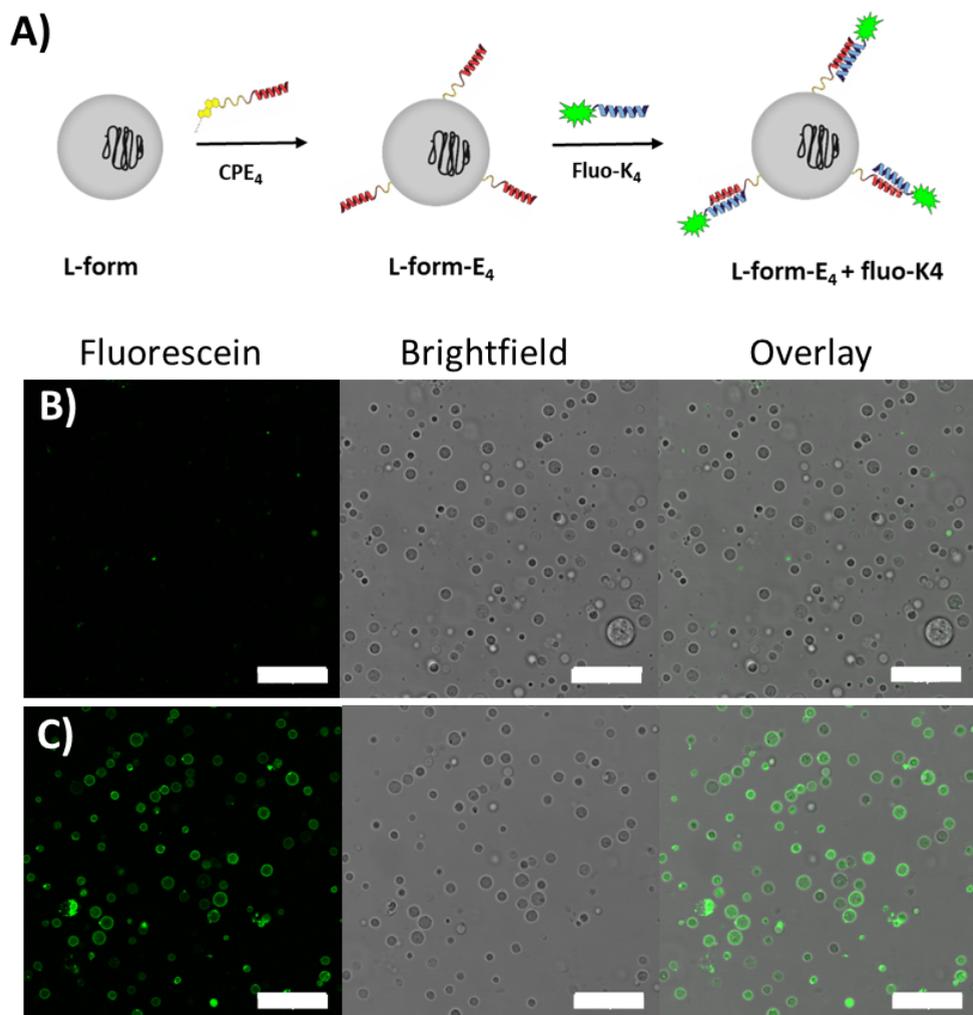


Figure 2. L-form membrane labelling assay. A) Scheme to illustrate the L-form membrane labelling process, B) L-form + fluo-K₄, C) L-form-E₄ + fluo-K₄. Scale bar: 20 μ m.

3. Coiled-coil peptide triggered L-form cell-cell membrane fusion

Next, the possibility of using the PK₄/CPE₄ coiled-coil pair to trigger L-form cell-cell membrane fusion was investigated. If HygG and AprR phenotypes fuse and resulting in content mixing, the fused L-forms should contain both GFP and mCherry. Additionally, these L-forms should be resistant to both antibiotics.

To induce fusion, HygG and AprR were both decorated with CPE₄ (Scheme 1). Peptide PK₄ was subsequently added to a mixture containing an equal number of

HygG and AprR cells. Due to coiled-coil formation between peptide PK₄ and the CPE₄ bound to the L-forms, this mixture should ultimately result in fusion. Since bacteria can take up free DNA from the surroundings, DNase was added to the medium in order to degrade DNA released from lysed L-forms to prevent it from being taken up by other cells, resulting in a false positive strain that is resistant to both antibiotics. This precautionary measure was taken as PK₄ has a high membrane affinity and can destabilize membranes facilitating membrane fusion (see Chapter 4 of this thesis), but also potentially induces lysis. Successful membrane fusion events were imaged using confocal microscopy to search for fused, doubly-fluorescent cells. Indeed, orange cells were obtained in the L-form mixture, suggesting that membrane fusion was achieved (Figure 3A). Due to the fact that HygG and AprR were both decorated with CPE₄, the addition of PK₄ not only induces fusion between L-forms with different markers but also between L-forms with the same markers, resulting in a low ratio of fused cells containing resistance to both antibiotics.

To confirm the viability of the fused L-forms and to calculate the efficiency of cell-cell fusion, the L-forms were transferred to an agar plate containing both antibiotics (apramycin and hygromycin). Fusion efficiency was calculated by counting the number of colonies on the double antibiotic selection plates divided by the number of AprR or HygG colonies obtained on single antibiotic selection plates. As a control, AprR and HygG were mixed in a 1:1 ratio and directly plated on double selection media. After four days' culture, colonies were found on the plates of the experimental group while no colonies were found on the plates of the control group. Analysis of the colonies showed that approximately 1 in 100,000 cells is fused. A range of PK₄ concentrations were subsequently tested and the results show that, with a higher concentration of PK₄, a higher fusion efficiency was achieved, (Figure 3B). Control experiments showed that even higher concentrations of PK₄ led to more L-form lysis (Figure 3C,3D & 3E), and therefore we decided not to use concentrations > 50 µM.

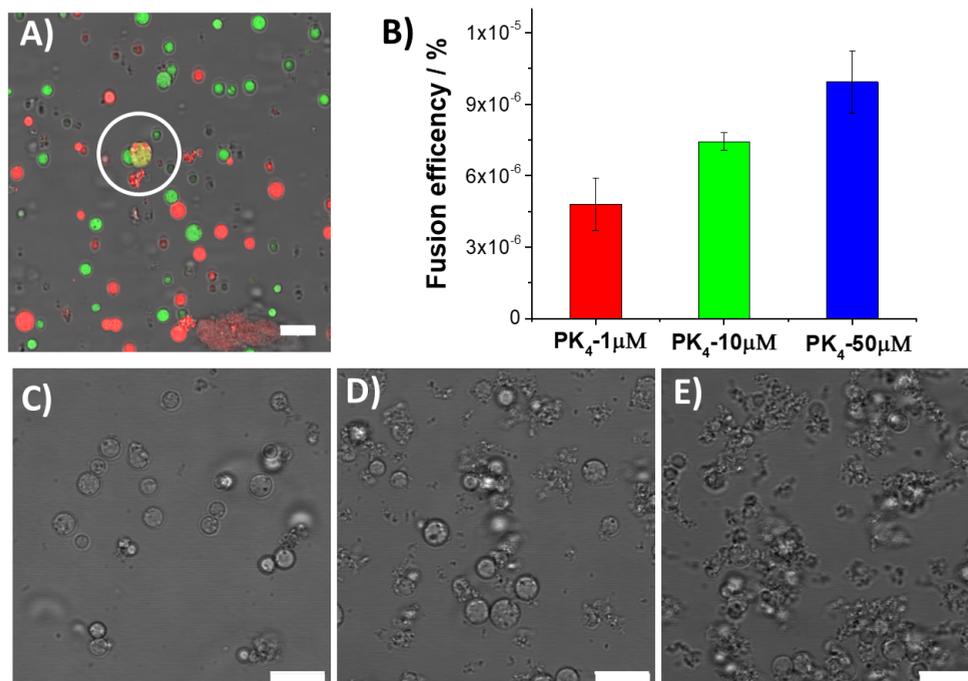


Figure 3. A) L-form mixture after cell-cell fusion, Green: GFP, Red: mCherry. B) L-form fusion efficiency is dependent on PK₄ concentration. L-forms with different concentrations of PK₄: C) 1 μM, D) 10 μM and E) 50 μM. Scale bar: 10 μm.

Next, we selected a colony from the double antibiotic-containing agar plate with a diameter of around 1.5 mm. The colony contained both green and red L-forms, as observed with confocal microscopy (Figure 4A). More red L-forms were found close to the centre and green L-forms were distributed at the edges of the colony. The shape of the colony is approximately round, which suggests that all daughter cells in the colony come from one mother cell. This mother cell must contain two sets of antibiotic resistance genes so that it survives on the double-antibiotic plate, and the daughter cells must also contain both genes as they survived. Additionally, the mother cell should contain both GFP and mCherry genes as both green and red L-forms were found in the colony. Unfortunately, no significant regions of orange were observed, as we expected after fusion. Interestingly, a few orange L-forms were observed on one edge of the colony (Figure 4B). L-forms typically contain multiple chromosomes due to the absence of a cell wall, which regulates chromosome segregation during division. After cell-cell fusion, the fused L-forms contain both sets of chromosomes which allow the L-form to exhibit orange fluorescence and to survive on the double antibiotic plates. After cell division, it

appears that two kinds of chromosomes contain different fluorescent protein genes but that the antibiotic resistance genes can go to the same, or different, daughter cells. Thus the daughter cells that survive on the plate show either single fluorescence or are doubly fluorescent and thus appear orange. Multiple mechanisms can explain the coexistence of these only green and red regions in the colony. First is the possibility of recombination of the resistance genes resulting in one chromosome containing both resistance markers but only one fluorescence marker. A second reason could be due to the atypical division process wherein the daughter cell remains connected to the mother cell during growth. This could result in unequal distribution of cytoplasmic material. Thirdly, differential expression of the fluorescence genes in the cell could occur due to competition between transcription factors and regulatory elements. The same reasoning can be provided for an unequal distribution of the chromosomes within a given cell resulting in the dominant chromosome providing the phenotype green or red. This aspect requires time-lapse imaging of a single colony to follow gene expression over time. Lastly, the cells could be exchanging cytoplasmic contents over short ranges to create an alternating green and red pattern. Some of these mechanisms are dependent on the environment (*i.e.* solid media) they are grown in and can be tested by changing to a more homogeneous environment with agitation (*i.e.* liquid media).

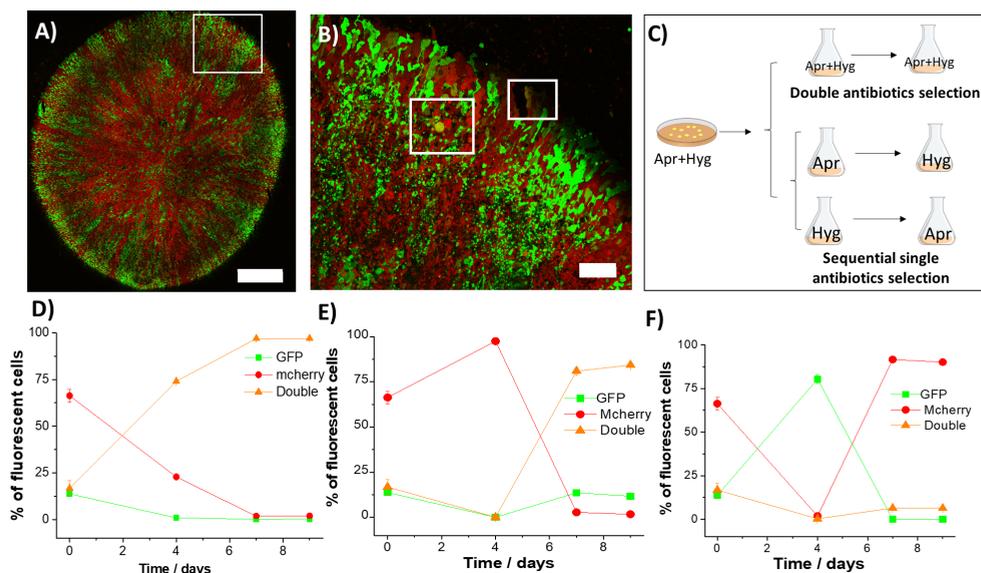


Figure 4. Image of a colony on a double antibiotic agar plate: (A) the whole colony, scale bar 300 μm ; (B) zoom in on the edge of the colony, scale bar 50 μm . (C) The scheme illustrates fused L-form enrichment. FACS of L-form populations during fused L-form enrichment with double antibiotics (D), or sequential single antibiotics selection: (E) Apr + Hyg, (F) Hyg + Apr.

4. Fused L-form enrichment by antibiotic selection in liquid media

This study shows that solid-state antibiotic selection is not good enough to obtain a high ratio of doubly fluorescent L-forms as predominantly green and red L-forms were found in the colony. As described above, L-forms growing close to each other on solid media could provide protection (by exchanging some cytoplasmic material) and create daughter cells, which survive in the presence of both antibiotics. To verify this hypothesis, we picked colonies from the agar plate and resuspended them in liquid medium. Two antibiotic selection strategies were then applied (Figure 4C). For the first selection strategy, an L-form suspension was cultured in media with double the concentration (apramycin: 100 $\mu\text{g}/\text{mL}$, hygromycin B: 200 $\mu\text{g}/\text{mL}$) of both antibiotics. FACS analysis and cell imaging were performed at different time points (0, 4, 7 and 9 days) after inoculation. More red than green L-forms were found from the picked colonies (Figure 4D & 5A). After 4 days of selection, the ratio of both red and green L-forms showed a large decline and 74% of doubly fluorescent L-forms were observed (Figure 4D & 5B). On day 7, the ratio of doubly fluorescent cells increased to 97% with less than 2% of red cells

and 1% of green cells (Figure 4D & 5C). These L-forms continued to grow in media containing both antibiotics for another two days and no obvious change in the ratios was found (Figure 5D).

The other strategy employed was sequential antibiotic selection, wherein the inoculated L-forms were cultured in one antibiotic at a double concentration for 4 days, followed by changing to the other antibiotic at a double concentration and continuing culturing for several days. Flow cytometry was used to quantify L-forms expressing different fluorescent proteins at different time points. Two different results were obtained in the sequential antibiotic selection approach. Double fluorescent L-form enrichment was achieved by first culturing the L-forms in apramycin (100 $\mu\text{g/ml}$) for four days then switching to hygromycin B (200 $\mu\text{g/ml}$). After enrichment, the doubly fluorescent L-forms reach 84% (Figure 4E). Conversely, when L-forms were first cultured in the presence of hygromycin B and then apramycin, only 6% of doubly fluorescent L-forms were found and more than 90% of the L-forms show only red fluorescence (Figure 4F). These results show that the double antibiotic selection strategy is better than sequential antibiotic selection to obtain fused L-forms enrichment. The enriched cells not only show double antibiotic resistance but also contain both fluorescent proteins. This selection experiment in liquid medium also confirms that cell-cell fusion was obtained rather than recombination of the antibiotic resistance genes.

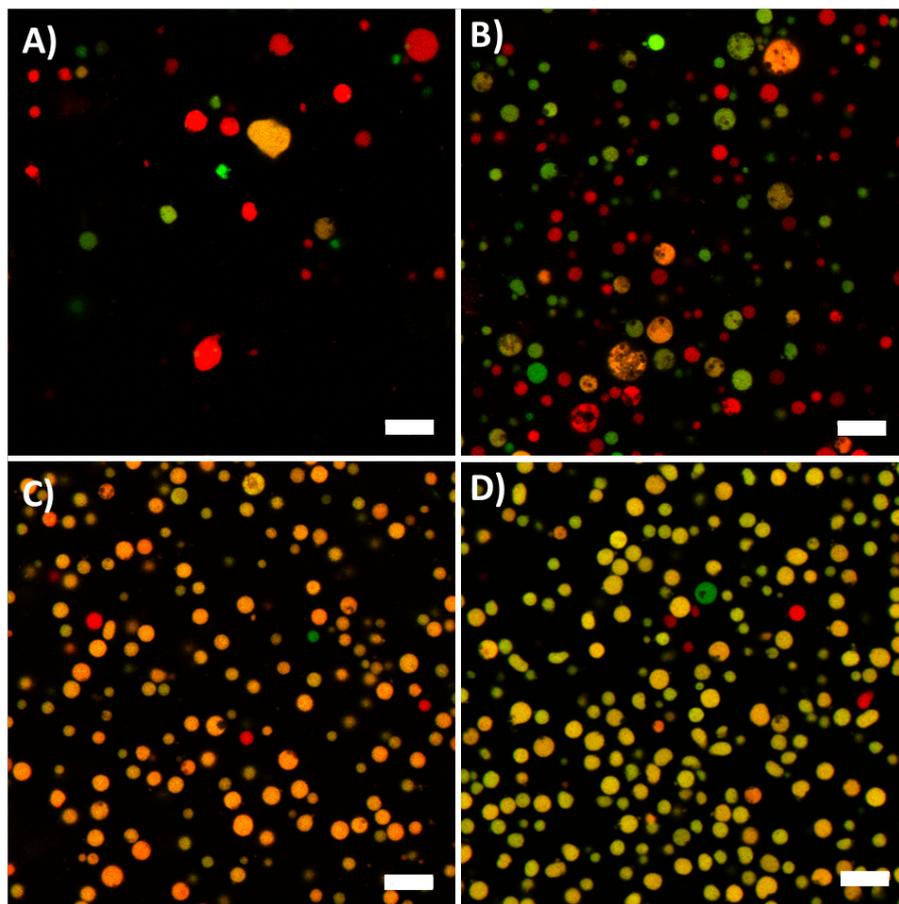


Figure 5. Fused L-form enrichment by double antibiotic selection at a double concentration at differing time intervals: (A) 0 days, (B) 3 days, (C) 7 days and (D) 9 days. Scale bar: 10 μm .

5. Tracking cell division of fused L-forms by time-lapse confocal microscopy and colony imaging

Cell-cell fusion results in the mixing of cytoplasmic and genomic contents. After several generations of double antibiotic selection, dual-labelled, and double antibiotic resistant L-forms with >97% purity were obtained. To study the viability growth and division of these fused L-forms, time-lapse confocal microscopy was employed. For this experiment, the L-forms were grown in media with double antibiotics. Figure 6 shows the process of L-form division for 12.5 hours. Round shaped L-forms were present at the start of the time-lapse experiment. After 100 min of incubation at 30°C, characteristic membrane deformation and blebbing were

observed. Gradually, daughter cells formed, still attached to the mother cells. Due to the production of daughter cells, the mother L-forms became smaller over time. Ultimately, the mother cells disappeared and divided into several daughter L-forms. Given the non-binary nature of cell division in wall-deficient cells, it was difficult to track the exact number of daughter cells originating from one mother cell. The newly formed daughter cells were typically producing more GFP than mCherry, suggesting that GFP is expressed more easily. Furthermore, after prolonged imaging, the fluorescence faded due to photobleaching. Because culturing L-forms in the confocal well plate (no shaking, less media) is not possible, we did not manage to obtain the nuances of cell division using the current set up.

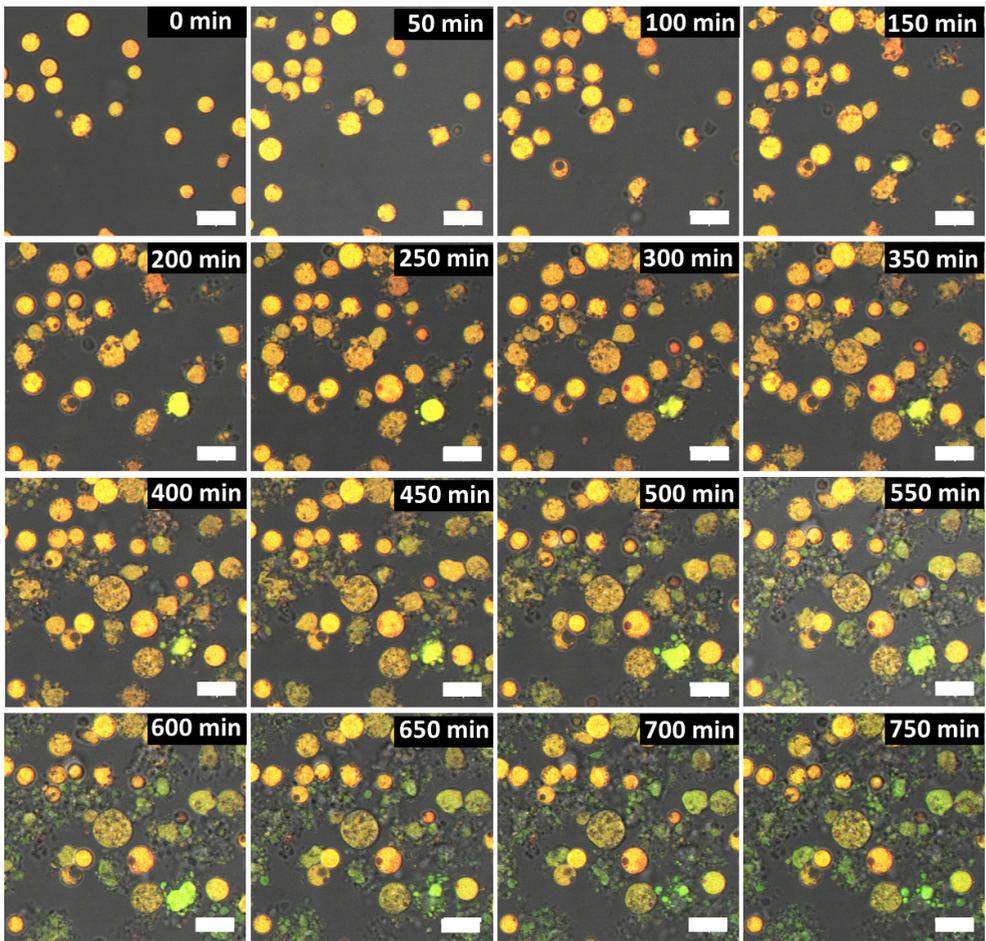


Figure 6. Time-lapse study of fused L-form division. Scale bar: 10 μ m.

The fused L-forms contain two sets of chromosomes with a different number of

copies. One set of chromosomes contains GFP and Hyg resistance genes while the other set of chromosomes contains mCherry and Apr resistance genes. With both antibiotics present during L-form culturing, only cells that contain both sets of chromosomes survive and produce daughter cells. Without antibiotics, there is a chance that the daughter cells only contain one set of chromosomes due to the unregulated chromosome segregation during cell division. To find evidence of chromosome segregation, the enriched L-forms were seeded on a solid agar plate without antibiotics. Chromosome segregation in this scenario will result in regions of the colony expressing either only green or only red fluorescence. To our surprise, a perfect orange colony was obtained (Figure 7A). The color of the whole colony is not homogenous. On the edge of the colony, some L-forms with only green or red fluorescence was observed, (Figure 7B), but the percentage of these is low compared to the orange cells. This suggests that fused L-forms are very stable after double antibiotic enrichment and chromosome segregation is difficult to achieve in short culturing regimes in the solid-state even when no antibiotics are present. In addition, a yellow-green ring was found on the outermost part of the colony. This result is consistent with the data in the time-lapse study of L-form culturing that shows yellow-green cells were formed after cell division. This suggests that GFP may be expressed before mCherry in newly formed L-forms. Another reason for slow segregation in the absence of selection is that there might be too many copies of each set of chromosomes in the fused L-forms after enrichment. A higher number of chromosomes would then require a longer time for segregational loss.

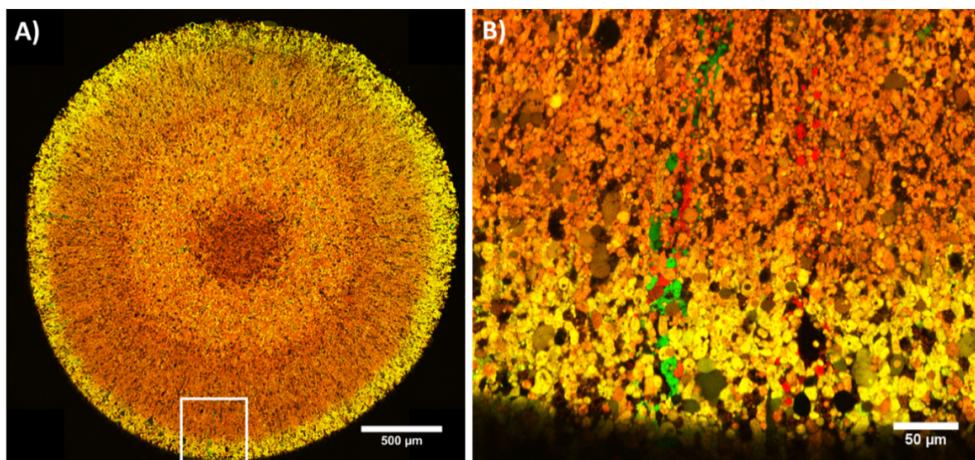


Figure 7. Imaging of fused L-form cultures on an antibiotic-free agar plate. A) The whole colony (scale bar: 500 μ M) and B) the edge of the colony (scale bar: 50 μ M).

CONCLUSION

In this work, we achieved fusion between L-forms using coiled-coil peptides for the first time. For this study, fluorescent protein markers and antibiotic markers were introduced into L-forms of *Kitasatospora viridifaciens* and two different strains (AprR and HygG) were produced. By performing an L-form membrane-labelling assay, we verified that CPE₄ incorporates spontaneously into L-form membranes and is able to form coiled coils with the complementary peptide K₄. The addition of the parallel dimeric peptide PK₄ to CPE₄ decorated L-forms resulted in fusion. Imaging of colonies after fusion revealed the presence of green, red, and orange L-forms, suggesting that gene-expressing in fused L-forms is unstable. L-form enrichment was achieved by culturing L-forms from colonies on double antibiotic selection media. After cell enrichment, 97% of the L-forms obtained contained double fluorescent protein markers as well as double antibiotic resistance markers. Division of fused L-form was studied by time-lapse imaging. Cell division was observed and occurred mainly by blebbing, showing the viability of the fused L-forms. We also studied chromosome segregation of the fused L-forms by culturing on an agar plate without antibiotics. After enrichment, the fused L-forms were found to be very stable and chromosome segregation was slow. In future experiments, we will test the biosynthetic capabilities of the fused cells compared to their parental strains. This study may open up a new field of genetic recombination by cell-cell fusion, which will have potential applications in biotechnological applications (*e.g.* novel antibiotics) as well as a model for the evolution of early cells (*i.g.* origin of life study).

EXPERIMENTAL SECTION

Media and L-form strains

L-phase broth (LPB) was used to culture L-forms in the liquid state. LPB contains 0.15% yeast extract, 0.25% bacto-peptone, 0.15% oxoid malt extract, 0.5% glucose, 0.64M sucrose, 1.5% oxoid tryptic soy broth powder (all w/v) and 25mM MgCl₂. The L-form culture was grown in a 50 mL flask containing 10 mL LPB and incubated at 30 °C with 100 rpm shaking.

L-Phase Medium (LPMA) was used to support the L-form growth in the solid state. It contains 0.5% glucose, 0.5% yeast extract, 0.5% peptone, 20% sucrose, 0.01% MgSO₄·7H₂O and 0.75% Iberian agar (all w/v). After autoclaving, the medium was supplemented with MgCl₂ to 25 mM and horse serum to 5% (v/v).

The P-buffer contains sucrose 10.3%, K₂SO₄ 0.025%, MgCl₂·6 H₂O 0.2%, , KH₂PO₄ (0.005%), CaCl₂·2 H₂O (0.368%) ,TES buffer (0.573%). TES buffer contains ZnCl₂ 36μM, FeCl₃·6H₂O 74μM, CuCl₂·2H₂O 5.8μM, MnCl₂·4H₂O 5μM, Na₂B₄O₇·10H₂O 2.6μM and (NH₄)₆Mo₇O₂₄·4H₂O 0.8μM. The P-buffer was used in cell membrane labelling assays, cell-cell membrane fusion assays and in FACS studies.

All L-form strains used in this study were obtained from the lab of Molecular Biotechnology, Institute of Biology, Leiden University. The *Kitasatospora viridifaciens* L-forms were made as previously reported.⁵ The GFP expressing phenotype HygG was made by introducing insertion plasmid pIJ82-eGFP which contains the gene *hph* (confers resistance to hygromycin B) into the wild type L-form. The mCherry expressing phenotype AprR was made by transforming the pSet152-mCherry plasmid which contains the gene *aac(3)IV* (confers resistance to apramycin). All genes are under constitutive expression and inserted in the same site (*attB*) on the chromosome. The plasmids were introduced into L-forms via PEG-assisted transformation.²⁵ L-form cultures were grown for 4 days. Cultures were centrifuged to remove the spent media and the pellet was resuspended in 1/4th volume P-buffer. Approximately 500 ng plasmid was added to the resuspended pellet and mixed thoroughly. PEG1000 was added to this mix at a final concentration of 25 w% and mixed gently. After a brief incubation of 5 minutes on the bench, the tube was centrifuged. The supernatant was discarded and the pellet was resuspended in LPB medium and incubated for 2 hours. The culture was then centrifuged again and the pellet resuspended in 100 μL LPB for plating on LPMA media containing selective antibiotics apramycin or hygromycin. After 4 days of incubation single colonies were picked and restreaked on LPMA with antibiotics for confirmation along with fluorescence microscopy.

Minimum inhibitory concentration (MIC) determination

LPMA plates containing different concentrations of antibiotics were prepared. The concentrations of apramycin used were 0, 2, 4, 10, 25 and 50 μg/ml whereas hygromycin was used at 0, 2, 10, 20, 50 and 100 μg/ml. A 3-day old L-form culture was serially diluted and the dilutions were used for spotting 10 μl onto each plate. The serial dilution was done to determine the MIC. The plates were incubated at 30°C for five days before colony counting was performed to determine the CFU/ml.

Peptides synthesis and stock solution preparation

The synthesis and purification of all peptides used in this chapter are described elsewhere in this thesis (**Chapter 3** and **Chapter 4**). Stock solutions of CPE₄ and PK₄ were prepared in DMSO at a concentration of 10 mM and 1 mM respectively. Fluo-K₄ was dissolved in H₂O at a concentration of 200 μM.

L-form membrane labelling

Wild type L-forms (3×10^8) were suspended in 1 ml of P-buffer (OD₆₀₀ = 0.4). 10 μL of CPE₄ stock solution was added to the L-form suspension to obtain a final concentration of 100 μM. After 30 min of incubation at 30 °C with shaking at 100 rpm, the L-forms were washed two times, by centrifugation, using P-buffer. The L-forms were then suspended in 900 μL P buffer and 100 μL of fluo-K₄ was added to a final concentration of 20 μM. After 5 min of incubation, the L-forms were washed three times using P-buffer to remove free fluo-K₄. L-form imaging was performed on a Leica SP8 confocal microscopy. Excitation: 488 nm, emission: 500-550 nm.

L-form cell-cell membrane fusion

L-form fusion was performed in P-buffer containing DNase (1 mg/mL) to ensure that all extracellular DNA present in the culture (due to cell lysis) is degraded and the resistance genes are unavailable for cellular uptake. 3×10^8 of AprR and HygG (1:1) cells in 1 ml of P-buffer (OD₆₀₀ = 0.4) were incubated with CPE₄ at 100 μM for 30 min at 30 °C. The L-forms were washed with P-buffer twice after incubation by centrifugation at 1000 g and resuspended in 1 mL of fresh P-buffer. PK₄ was added to the L-form suspension to a concentration of 1, 10 or 50 μM. After 10 min of incubation, the L-forms were centrifuged (1000 g) and washed once before seeding on agar plates containing both antibiotics. The assay was performed in quadruplicate. After cell-cell membrane fusion, cell imaging was performed on a Leica SP8 confocal microscopy. Channels: GFP excitation: 488 nm, emission: 500-550 nm; mCherry excitation: 535 nm, emission: 550-600 nm.

Cell-cell membrane fusion efficiency calculation and colony imaging

Cell-cell fusion efficiency was calculated by counting CFU of colonies on double antibiotic selection (fused cells) and single antibiotic selection (parent cells) plates. The efficiency of fusion was calculated by counting the number of colonies on double antibiotic selection plates divided by the number of AprR or HygG colonies

obtained on single antibiotic selection plates. As a control, AprR and HygG were mixed in a 1:1 ratio and directly plated on double selection media.

A colony of ~1.5 mm diameter was picked up by cutting the gel and placing it upside down on the ibidi μ -Slide 8-well plate. The colony was plastered to the bottom surface of the slide. Colony imaging was performed on a Leica SP8 confocal microscope. In order to image the whole colony, the tile scan function was used. For each tile, five layers were recorded. The overall colony image was obtained by merging all the tiles. Channels: GFP excitation: 488 nm, emission: 500-550 nm; mCherry excitation: 535 nm, emission: 550-600 nm.

Time-lapse confocal imaging

Time-lapse confocal imaging was performed also using a Leica SP8 confocal microscope equipped with a temperature controllable incubator set at 30 °C. 3×10^5 L-forms were seeded in ibidi μ -Slide 8 Well plates with 300 μ L LPB. The samples were placed on the stage for 30 min in order to make the flowing L-forms sediment to the bottom of the plate before imaging. A low laser power (0.5% for 488 nm and 0.2% for 535 nm) and high gain (750 for both channels) was used in order to obtain high-quality images as well as to prevent fluorescent protein bleaching. L-form were imaged every 5 min for 13 hours using a 63 \times oil immersion objective.

L-form Enrichment

Approximately 30 colonies from the double antibiotic selection plate were picked and resuspended in 1.5 mL LPB. The fused L-form enrichment was performed in 50 ml flasks with 10 mL of LPB. For double antibiotic selection, the medium contained 100 μ g/mL Apr and 200 μ g/mL Hyg. L-forms were grown in an incubator with 100 rpm shaking at 30°C. L-form subculture was performed after 4 days and 7 days by transferring 200 μ L L-form suspension to a new flask containing the same amount of LPB and antibiotics. For sequential single antibiotic selection, L-forms were inoculated in a flask containing either 100 μ g/mL Apr and 200 μ g/mL Hyg. After 4 days culturing, the L-forms were subcultured by transfer to a new flask containing different antibiotics to the inoculated flask. After 3 days, the L-forms were subcultured again in a new flask with the same antibiotics. Fluorescence assisted cell sorting (FACS) was used to quantify the L-form population.

FACS

L-form cultures from different time points were first washed twice by centrifugation and resuspended in P-buffer ($\sim 1 \times 10^5$ cell/ml) to remove the background arising from the LPB (yellow-coloured medium) and green pigment produced by the L-forms. Next, 100 to 200 μL of culture was analyzed by FACS. The S3e Cell sorter (Bio-Rad) works by generating droplets of the culture such that each droplet consists of roughly 1 cell or particle. This droplet passes through a laser to excite the fluorescent proteins inside the cell. Using a combination of lasers (488 and 561 nm) and emission filters (FL1: 525/30nm and FL2: 586/25) the presence of multiple fluorescent proteins inside a single cell was detected. For all cultures, approximately 20,000 events were tested with 2 technical replicates. The readout obtained was a signal for either GFP or mCherry that was displayed as a scatter plot with the green and red channels as axes. The graph was then divided into 4 quadrants namely green, red, double label and no label. Percentages of cells within the population that fall into each category was obtained using the ProSort™ software version 1.6.

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