Heterologous protein transfer within structured myxobacteria biofilms

Xueming Wei, Darshankumar T. Pathak and Daniel Wall*
Department of Molecular Biology, University of Wyoming, 1000 E. University Ave., Laramie, WY 82071, USA.

Summary

Microbial biofilms represent heterogeneous populations of cells that form intimate contacts. Within these populations cells communicate, cooperate and compete. Myxobacteria are noted for their complex social interactions, including gliding motility and lipoprotein exchange. Here, we investigated cis protein sequence and cellular behaviour requirements for lipoprotein transfer between Myxococcus xanthus cells. Specifically, an outer membrane (OM) type II signal sequence (SS) fused to the heterologous mCherry fluorescent reporter resulted in OM localization. When donor cells harbouring SS_{OM–mCherry} were mixed with GFP-labelled recipient cells they developed red fluorescence. Our results surprisingly showed that a type II SS for OM localization, but not inner membrane localization, was necessary and sufficient for rapid and efficient heterologous protein transfer. Importantly, transfer did not occur in liquid or on surfaces where cells were poorly aligned. We conclude that cell–cell contact and alignment is a critical step for lipoprotein exchange. We hypothesize that protein transfer facilitates cooperative myxobacteria behaviours.

Introduction

Myxobacteria are Gram-negative soil microbes that exhibit complex social behaviours. Central to these behaviours is gliding motility, which powers and coordinates swarm expansion, rippling, predation and fruiting body development on solid surfaces. Myxococcus xanthus has two motility systems called A (adventurous) and S (social) motility (Hodgkin and Kaiser, 1979a). Mutations in one of these systems block the corresponding motility system; however, the cells remain motile as the other system is intact. Thus non-motile mutants (A–S–) typically contain two mutations. Of interest here, a subset of motility mutants can be complemented extracellularly when mixed with another strain that contains the corresponding wild-type gene (Hodgkin and Kaiser, 1977; 1979b). This process was called ‘stimulation’ as the recipient mutant transiently gains the ability to glide. Stimulation only involves phenotypic changes; there are no genotypic changes. Of these six motility genes (cglB/C/D/E/F and tgl) two have been identified; cglB (A-motility) and tgl (S-motility) (Rodriguez-Soto and Kaiser, 1997; Rodriguez and Spormann, 1999). Both of these genes contain type II signal sequences and thus encode lipoproteins. The mechanism for stimulation involves cell–cell transfer of either the CglB or Tgl lipoproteins from donor to recipient cells (Nudleman et al., 2005). Thus, mutant recipient cells obtain these proteins from donor cells and consequently the corresponding protein activity allows gliding motility for a transient period of time. Strikingly, lipoprotein transfer was very efficient. The biological purpose and molecular mechanism of transfer are currently unknown.

Cellular exchange of proteins is broadly found in the bacterial and eukaryotic kingdoms. The type III secretion system is one of the best described systems used by bacterial pathogens to inject effector proteins into eukaryotic host cells. Other bacterial cell–cell contact mediated delivery systems, including type IV, V and VI, transfer proteins either within or between kingdoms (Hayes et al., 2010). These systems typically transfer a toxin or are part of a conjugative system. Protein transfer implicated by bacterial outer membrane vesicles (OMVs) represents a distinct mechanism that does not appear to involve direct cell–cell contact (Kulp and Kuehn, 2010). Nanotubes were recently discovered in a Gram-positive bacterium to mediate cytoplasmic exchange of proteins within and between bacterial species (Dubey and Ben-Yehuda, 2011). Plant cells are also widely known to transfer proteins via their plasmodesmata (Oparka, 2004). Protein exchange between mammalian cells has received renewed attention, particularly between immune cells (Davis, 2007; Rechavi et al., 2009). Although the mechanisms and reasons for cellular protein exchange vary, its occurrence is widespread and important for diverse biological processes.
Myxobacteria are known to form dynamic biofilms on different surfaces. For instance, when placed on plastic, membrane or agar surfaces they quickly adhere and produce an extracellular matrix (Kuner and Kaiser, 1982; Arnold and Shimkets, 1988; Dworkin, 1999; Palsdottir et al., 2009). These motile cells rapidly align and exchange proteins (Wall and Kaiser, 1998; Nudleman et al., 2005). Depending on environmental cues their biofilms might swarm, ripple or erect fruits (Igoshin et al., 2004). To initiate studies of myxobacteria protein transfer, we sought to define cis protein sequences within Tgl and CglB that allows heterologous protein transfer. We report here that a type II signal sequence for OM localization was sufficient for heterologous protein transfer. This fluorescent reporter was then used to monitor cellular transfer dynamics.

Results

mCherry fusions localize to the IM or OM

To visualize the localization and transfer of the Tgl and CglB lipoproteins they were fused to mCherry. This reporter was chosen as it can properly localize and function when fused to a type I or II signal sequence in a wide range of Gram-negative bacteria (Chen et al., 2005; Lewenza et al., 2006; 2008). Consequently, a number of such Tgl and CglB fusions were made to the N- or C-terminus of mCherry. Although these constructs were fluorescent and showed envelope localization, they did not complement corresponding Tgl or CglB mutants, and Western blot analysis revealed the fusion proteins were cleaved when expressed in M. xanthus (data not shown). Therefore, we simply used a type II signal sequence fused to mCherry that was shown to result in OM localization in other bacteria (CSFP sorting sequence; Lewenza et al., 2008). This fusion, dubbed SS_{OM}-mCherry, was expressed from the pilA promoter in Escherichia coli and M. xanthus and was localized to the cell envelope (Fig. 1A and C, left panels). As a control, mCherry was also fused to a type II signal sequence shown to result in IM localization in M. xanthus (MXAN_1176 encoding peptidyl-prolyl isomerase with a CKDSDKKESM sorting sequence for IM retention; L. Shimkets and S. Bhat, pers. comm.).
Indeed, when expressed from P_{pilA} the SS_{OM}-mCherry reporter showed envelope localization in *E. coli* and *M. xanthus* (Fig. 1A and C, right panels). We note the SS_{IM}-mCherry reporter also displayed a punctuate pattern in about half of the examined cells (*M. xanthus* and *E. coli*). A similar punctuate pattern was seen, though rarely, with SS_{OM}-mCherry. Next, to distinguish between IM and OM localization cells were subjected to plasmolysis (Lewenza *et al.*, 2006; 2008). As predicted, following plasmolysis SS_{OM}-mCherry retained envelope localization in both *E. coli* and *M. xanthus*, indicating it resides in the OM as it was protected from membrane collapse by the rigid cell wall (Fig. 1B and D, left panels). In contrast, following plasmolysis SS_{IM}-mCherry localization changed. Instead, in both *E. coli* and *M. xanthus*, fluorescent cytoplasmic aggregates formed, indicating the IM collapsed with SS_{IM}-mCherry (Fig. 1B and D, right panels). We also note that fluorescent membrane material sloughed off plasmolysed *M. xanthus* cells expressing SS_{OM}-mCherry (Fig. 1D and E, right panels). We believe this material represents the OM, as no fluorescent extracellular material was found from plasmolysed *M. xanthus* cells expressing SS_{IM}-mCherry (Fig. 1D and E, right panels). Lastly, we found OMVs from cultures and purified preparations were readily seen by fluorescent microscopy with the SS_{OM}-mCherry construct. In contrast, no OMVs were seen with the SS_{IM}-mCherry construct (data not shown). Altogether, we conclude that SS_{OM}-mCherry and SS_{IM}-mCherry localize to the OM and IM respectively.

**Fig. 2.** Test for mCherry fusion transfer to motile *M. xanthus* recipients. Donor cells were non-motile (DW1047 and DW1048) and contain indicated reporters. The non-fluorescent recipient was A*S* (DK8615). Cells were spotted at a 1:1 mixture at Klett 250. Micrographs were taken at swarm edges where primarily motile cells (DK8615) were found after incubation for 22 h on 1/4 CTT agarose pad. Top panels (A and C) are phase contrast micrographs while the bottom panels (B and D) are the identical fluorescent fields (20X objective). Arrows mark an isolated motile *M. xanthus* cell in (A) and (B) where SS_{OM}-mCherry transfer occurred. *M. xanthus* cell dimensions are approximately 0.5 ¥ 6.0 microns.

A type II_{OM} signal sequence is necessary and sufficient for heterologous protein transfer

Prior studies showed the CgIB and Tgl lipoproteins were efficiently transferred between cells (Nudelman *et al.*, 2005). Here we tested whether heterologous mCherry reporters could be transferred. First, a non-motile donor strain that contained either the SS_{OM}-mCherry or SS_{IM}-mCherry was mixed with a motile non-fluorescent recipient at a 1:1 ratio. These mixtures were spotted on an agarose pad to allow protein transfer and gliding motility. After one day of incubation single and rafts of cells were seen swarming out from the inoculum spots (Fig. 2A and C). To test if the motile, non-fluorescent recipients had received the reporter from either donor the identical swarm edges were viewed by fluorescent microscopy. Here motile recipient cells present at the swarm edge showed bright fluorescence when the donors expressed SS_{OM}-mCherry (Fig. 2B). Importantly, fluorescent labelling included small groups and single cells, clearly indicating that distal motile cells were not mixed with non-motile fluorescent donors (Fig. 2A and B arrows). In contrast, when non-motile donors expressed SS_{IM}-mCherry the motile recipients at the swarm edge did not exhibit fluorescence (Fig. 2D), indicating SS_{IM}-mCherry was not transferred. To further test protein transfer a non-motile donor strain was used that expressed both SS_{OM}-mCherry and cytoplasmic GFP fluorescent reporters. This strain was mixed with a motile (A*S*) non-fluorescent recipient. At the swarm edge, where mostly motile cells reside, it was again found that
SSOM–mCherry transferred to motile recipient cells (Fig. 3A and B). In contrast, cytoplasmic GFP did not transfer (Fig. 3C). We also note that a low frequency (~1%) non-motile donor cells were pushed or dragged out to the beginning of the swarm edge by motile cells. As indicated by arrows, these donor cells were easily identified as their expressed GFP and mCherry fluorescence was brighter than that found in recipient cells (Fig. 3B and C). Regarding this latter point, the low level of SSOM–mCherry fluorescence seen in recipients can be explained as most of these recipients would not have contacted SSOM–mCherry donor cells for extend periods (h), thus their fluorescence intensity would be expected to be diluted by cell division and protein turnover (Fig. 3B). More striking was the presence of bright donors among dim recipients (Fig. 3B, arrows). This result suggests that protein transfer might be regulated, as constitutive protein exchange should result in equal fluorescent intensities among donors and recipients. In summary, these results indicate that SSOM–mCherry was readily transferred from donor to recipient cells, while SSIM–mCherry and cytoplasmic GFP were not transferred.

A second assay was employed to confirm the selective transfer of SSOM–mCherry. Here, to definitively identify recipients from a mixed population they were labelled with cytoplasmically expressed GFP, while donors again expressed either mCherry construct. These donor and recipients were mixed and when immediately viewed (t = 0) cells only fluoresced green or red; no cell was double labelled (data not shown). Next, these cell mixtures were spotted on a solid agar surface, harvested at 4 h, washed and then placed on a glass slide for examination. Again transfer was observed for SSOM–mCherry. That is, GFP labelled recipients fluoresced red when they were mixed with SSOM–mCherry donors (Fig. 4, left panel). As shown all GFP recipient cells (>20) exhibited red fluorescence. Interestingly, the fluorescent intensities in recipient cells nearly matched that of donor cells indicating that SSOM–mCherry transfer was very efficient (Fig. 4, red left panel). Upon careful examination the recipient cells also showed envelope localization of SSOM–mCherry, indicating transfer resulted in the same subcellular localization as found in donors (Fig. 4, red left panel inset). As a control, when the same recipients were instead mixed with SSOM–mCherry donors, they only showed green fluorescence (Fig. 4, right panel). We further note that upon extended incubations and after examination of hundreds of recipient cells in no case was SSOM–mCherry transfer observed (data not shown). As found above, cytoplasmic GFP was not transferred (Figs 3C and 4). These results thus show that a type II signal sequence for OM localization was necessary and sufficient for heterologous protein transfer between M. xanthus cells.
Transfer requires a solid surface

The retraction of type IV pili (Tfp) provides the force to pull cells forward during S-motility (Wall and Kaiser, 1999). Prior studies of Tgl stimulation of Tfp biogenesis found that a solid surface was required for stimulation, as judged by Tfp biogenesis and S-motility (Wall and Kaiser, 1998). Here, we directly tested whether SSOM–mCherry transfer occurred in liquid by using the above assay and simply counting the number of recipients that turned red. As shown in Fig. 5, when donor (SSOM–mCherry) and recipient (GFP) cells were mixed and shaken in a culture flask no transfer was observed after 4 h. As a positive control, when the identical cell mixtures were placed on a 1.2% agar surface efficient transfer occurred (Fig. 5). Prolonged incubations, 24–72 h, either with shaking or static cultures, where several hundred recipients were examined, similarly resulted in no SSOM–mCherry transfer (data not shown). Therefore, as found for Tgl stimulation, protein transfer does not occur at detectable levels in liquid. To extend these findings and address the consideration that cells may not be at a sufficient density or
perhaps were not in the act of gliding, the identical cell mixtures were instead spotted on a soft (0.3%) agar plate. Again, as found for Tgl stimulation (Wall and Kaiser, 1998), no SSOM–mCherry transfer was observed at 4 h (Fig. 5). We note, however, that after prolonged incubations and growth, e.g. a day, some transfer was eventually detected between cells on the biofilm surface on soft agar (data not shown). We therefore conclude that a relatively hard surface was required for efficient SSOM–mCherry transfer.

The role of cell motility in transfer kinetics

Previously, we found that cell motility and alignment significantly enhanced Tgl stimulation (Wall and Kaiser, 1998). Since cell alignment particularly enriches end-to-end cell contacts and the Tfp and pil prophage, including Tgl, are polarly localized, we concluded stimulation and lipoprotein exchange occurred between cell poles (Wall and Kaiser, 1998; Nudleman et al., 2005; 2006; Bulyha et al., 2009). Here, the role of cell motility in heterologous protein transfer of a symmetrically distributed OM reporter was tested (Fig. 1). The rate and efficiency of protein transfer was thus measured between strain mixtures with varying degrees of A- or S-motility. For these experiments strains were mixed, spotted on a hard agar surface, and harvested at various times for microscopic examination of SSOM–mCherry transfer. As a preamble for the sequence of events we found that: (i) the inoculation spot dried in ~5 min, (ii) robust cell motility occurred after ~20 min, and (iii) transfer was first detected by ~1 h (data not shown). For strain comparisons, Fig. 6A shows that when the donor was non-motile (A–S–) and recipients (GFP+) were endowed with either A- or S-motility, SSOM–mCherry transfer occurred rapidly and efficiently, as ~90% of recipients acquired the reporter within 2 h. No discernable dif-
ference in transfer was observed between these motile strains (Fig. 6A). Similar transfer rates and efficiencies were found when donors were instead endowed with A- or S-motility or when both recipient and donors were motile (Fig. 6B). In contrast, when donor and recipients were non-motile virtually no SSOM–mCherry transfer occurred; even after prolonged incubations (Fig. 6A and B). Importantly, as controls, these identical donor and recipient strains were competent for transfer when mixed with a motile partner (Fig. 6A and B), thus the transfer defect was not an idiosyncratic property limited to specific strains. Interestingly, an intermediate SSOM–mCherry transfer rate was seen when a non-motile donor was mixed with a non-motile, but stimulatable recipient (A- tgl– gfp+; Fig. 6A). For this latter experiment both strains were initially non-motile, but over the two-day incubation period recipient cells were ‘stimulated’ to make Tfp and consequently slowly gained S-motility (Wall and Kaiser, 1998). This delayed, or slow development of S-motility, correlated with delayed SSOM–mCherry transfer kinetics, which after 2 days measured >90% transfer to recipient cells (Fig. 6A). Lastly, to test the hypothesis that motility simply provides a mechanical means to align cells, a transfer experiment was conducted between non-motile donor (SSOM–mCherry) and recipient (GFP+) cells mixed 1:1:1 with a third non-fluorescent motile strain (A+S–). Importantly, efficient and rapid SSOM–mCherry transfer occurred (Fig. 6A). These results show that motility plays a critical, but indirect role in protein transfer. That is, there was no requirement for A- or S-motility in donor or recipient cells, rather motility was only required in one cell type or in a third party strain for transfer to occur.

Motility aligns cells

The above results suggest that cell motility may facilitate protein exchange by allowing cell alignment between donor-recipient cells. Here, fluorescent microscopy was used to directly observe the effect of motility and surface conditions on donor-recipient cell alignment. Numerous conditions were tested and three are shown (Fig. 7). In all cases the identical non-motile donor (A-S–; SSOM–mCherry) and recipient (A+S+; GFP) cells were mixed. When a third non-fluorescent motile strain (A+S+) was added (1:1:1 ratio) and placed on a hard agarose surface, non-motile fluorescently labelled donor and recipient cells became highly aligned (Fig. 7A and B and inset). Evidently, the non-fluorescent A-motile cells pushed and dragged non-motile fluorescent cells into alignment. Figure 7B also reveals that cells are tightly packed and ordered in continuous streams. In contrast, when the same cell mixture was spotted on a soft agarose pad they did not align as cells sank in the porous matrix (Fig. 7E and F and inset). Similarly, in the absence of motile cells the non-motile fluorescently labelled cells poorly aligned on a hard agarose surface (Fig. 7C and D and inset). We conclude SSOM–mCherry transfer requires cell motility, which correlates with cell alignment and protein exchange efficiency (Wall and Kaiser, 1998; Wu et al., 2009).

Discussion

Myxobacteria exchange lipoproteins that can result in phenotypic changes to recipient cells without changing their genotype (Hodgkin and Kaiser, 1977; Wall and Kaiser, 1998; Nudleman et al., 2005). Our major new findings are: (i) the identification of cis protein sequences and (ii) cell motility requirement for heterologous lipoprotein transfer between *M. xanthus* cells, as diagrammed in Fig. 8. Surprisingly, we found that simply fusing a type II signal sequence for OM localization was necessary and sufficient for heterologous protein transfer. These findings, i.e. no other cis protein sequence was required for transfer, imply that other OM lipoproteins are shunted between *M. xanthus* cells (Fig. 8). As the *M. xanthus* genome contains nearly 400 predicted lipoproteins there are many candidate proteins for transfer (Goldman et al., 2006). We would expect, however, that some OM lipoproteins do not transfer because they are bound to a cellular complex, e.g. cell wall, or are sequestered away from a putative subcellular translocation site. Our results also show that protein transfer was rapid and efficient, occurring on a minutes to hours timescale. Since *M. xanthus* cells divide every ~5 h during log growth, protein exchange likely occurs frequently and repeatedly within a cells lifespan. Our findings also suggest that IM lipoproteins are not transferred. Whether soluble periplasmic or integral OM proteins are transferred is under investigation.

Our findings provide clues for the steps in transfer. Since SSOM–mCherry, Tgl and CglB all contain type IIOM signal sequences, we predict, based on the *E. coli* paradigm and bioinformatics, nascent polypeptides are transported across the IM in a Sec-dependent manner, followed by lipoprotein processing and Lol transport to the OM (Goldman et al., 2006; Ruiz et al., 2006). Once localized in the OM a number of transfer mechanisms are plausible. One mechanism, proposed nearly 30 years ago, suggests OMVs mediate transfer in *M. xanthus* (Helman, 1984). Although Helman provided some experimental support for this model, the findings were inconclusive. Recent proteomic and electron microscopy investigations have better defined *M. xanthus* OMV production, particularly within biofilms, and have revitalized the discussion that OMVs may mediate transfer (Palsdottir et al., 2009; Kahnt et al., 2010; Remis et al., 2010). This possibility builds on an expanding understanding that OMVs are universally produced by Gram-negative bacte-
ria and in some cases facilitate protein transfer (Kulp and Kuehn, 2010). However, our findings that transfer does not occur in liquid nor even in unstructured biofilms, but instead requires specific cell–cell contacts, suggest that protein transfer does not occur via diffusible OMVs. Since alignment of rod shaped cells particularly enriches end-to-end contacts, we envision that the site of transfer is at the cell poles (Wall and Kaiser, 1998). However, we recognize cell alignment can also enrich side-to-side contacts, which recently were shown to trigger intracellular receptor alignment (Mauriello et al., 2009), thus we cannot exclude their involvement. Aside from OMVs, direct OM fusion or a dedicated OM transport system could explain lipoprotein transfer. Recently, we have identified cellular proteins required for transfer and are currently investigating mechanisms.

**Fig. 7.** Motility aligns *M. xanthus* cells. Phase contrast micrographs (20x objective) are paired with identical field red/green fluorescent overlays. Cells were spotted at Klett 250 and inspected after 4 h. A and B. Non-motile fluorescently labelled cells (DK8606, GFP and DW1047, SSOM–mCherry) were mixed with a non-fluorescent A−S− strain (DK8615) at a 1:1:1 ratio on a hard (0.8%) agarose pad. C and D. Same as (A) and (B) except DK8615 was omitted. E and F. Same as (A) and (B) except strains were spotted on a soft (0.2%) agarose pad.

**Fig. 8.** Model for lipoprotein transfer between *M. xanthus* cells. Lollipops represent OM lipoproteins, e.g. SSOM–mCherry, Tgl and CglB. Upon aligned cell–cell contact OM lipoproteins are efficiently transferred to recipient cells. IM lipoproteins and cytoplasmic proteins are not transferred.
Why do myxobacteria exchange proteins? There are a number of possible explanations that are not mutually exclusive. First, protein exchange may serve a signalling role to provide spacial cues to co-ordinate cell behaviours. Direct cell–cell signalling is already known to orchestrate complex social behaviours such as fruiting body formation and rippling (Kaiser et al., 2010). Second, protein transfer might function in self/non-self recognition. Such discrimination apparently occurs as independent M. xanthus environmental isolate swarms do not merge, while kin swarms merge (Vos and Velicer, 2009). Third, protein transfer may represent a form of cooperative behaviour whereby kin cells share resources. Selective transfer between kin cells would ensure that non-kin cells do not exploit those resources (Velicer et al., 2000). Protein transfer would thus represent a logical and controlled extension of resource sharing whereby M. xanthus cells feed as cooperative microbial ‘wolfpacks’ (Rosenberg et al., 1977; Berleman and Kirby, 2009). Fourth, protein exchange may play a functional role in motility, development and/or other cellular processes. Indeed, protein exchange and functionality is detected with Tgl and Cgl mutants (Hodgkin and Kaiser, 1998). Thus myxobacteria may share parallels with eukaryotic cells, and understanding why and how proteins are exchanged will advance our knowledge of complex multicellular interactions.

### Experimental procedures

#### Strains and media

Bacterial strains used in this study are listed in Table 1. M. xanthus was routinely cultured in CTT medium (Hodgkin and Kaiser, 1977). As indicated 1/2 or 1/4 CTT media was used in which casitone levels were correspondingly reduced. M. xanthus cultures were grown in the dark at 33°C shaken at 350 r.p.m. and were generally harvested at ~100 Klett units (3 × 10^8 cfu ml^-1). TPM buffer contains 10 mM Tris, 1 mM potassium phosphate and 8 mM MgSO_4, pH 7.6. E. coli was

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culture in LB at 37°C (Sambrook and Russel, 2001). When necessary antibiotics were added: kanamycin (Km) at 50 μg ml⁻¹, oxy-tetracycline (oxy-Tc) at 12 μg ml⁻¹, streptomycin (Sm) at 250 μg ml⁻¹ for M. xanthus, and Km at 50 μg ml⁻¹, Sm at 100 μg ml⁻¹ or ampicillin at 100 μg ml⁻¹ for E. coli.

**Plasmids and strain construction**

Routine cloning manipulations were done following established protocols (Sambrook and Russel, 2001). PCR was done with Taq Master Mix (New England BioLabs) and products were routinely cloned into pCR2.1TOPO (Invitrogen, Carlsbad, CA, USA). The *mCherry* constructs used here were derived from pCHAP6656 (Lewenza et al., 2008), which contains a type II signal sequence for OM localization (CSFP; SSOM–mCherry) fusion was done by fusing the N-terminal end of mCherry at the XbaI site (Lewenza et al., 2008). The *pilA–SSOM–mCherry-R* fusion was also sub-cloned into pCR2.1 for identification of recipients. As described above donor and recipient strains were prepared. Alternatively, for liquid assays strains were prepared as described and transferred into a tube with 0.15 ml ½ CTT medium and incubated at 33°C without shaking. At various times cells were either scraped from agar plates or aliquoted from tubes, washed in TPM and pipetted onto a polylysine coated glass slides with a coverslip. Cells were observed with a Nikon Eclipse E800 microscope equipped with an array of objectives including a 100× 1.3 NA phase contrast oil submersion lens. Appropriate filter sets were used to visualize mCherry or GFP fluorescence. Images were captured with a Hamamatsu CCD camera and processed with Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA). To determine the per cent of recipients (GFP⁺) that received mCherry at least 50 and up to 200 cells were examined per sample or time point.

**Plasmolysis**

Plasmolysis was done as essentially described (Lewenza et al., 2006; 2008). Briefly, log phase cells were washed and resuspended in TPM buffer (control) or in plasmolysis solution (0.5 M NaCl). Control cells were mixed with melted 1% agarose pads (0.8 ml) prepared on glass slides and incubated at 33°C in a humid chamber. At various times cells were microscopically observed (20× objective). mCherry transfer was thus detected as emerging fluorescent flares from the inoculation spot, as donor cells were non-motile.

The second assay for mCherry transfer was as follows. Donors again expressed one of the mCherry reporters and recipients expressed cytoplasmic GFP that does not transfer (Wall and Kaiser, 1998). GFP allowed convenient identification of recipients. As described above donor and recipient strains were prepared. Alternatively, for liquid assays strains were prepared as described and transferred into a tube with 0.15 ml ½ CTT medium and incubated at 33°C without shaking. At various times cells were either scraped from agar plates or aliquoted from tubes, washed in TPM and pipetted onto a polylysine coated glass slides with a coverslip. Cells were observed with a Nikon Eclipse E800 microscope equipped with an array of objectives including a 100× 1.3 NA phase contrast oil submersion lens. Appropriate filter sets were used to visualize mCherry or GFP fluorescence. Images were captured with a Hamamatsu CCD camera and processed with Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA). To determine the per cent of recipients (GFP⁺) that received mCherry at least 50 and up to 200 cells were examined per sample or time point.

**Protein transfer between cells**

A swarm assay was first used to detect mCherry transfer. Here, non-motile and fluorescent donors (DW1047; *aglB1 ΔpilA PilA–SSOM–mCherry* or DW1048; *aglB1 ΔpilA PilA–SSOM–mCherry*) were mixed 1:1 with an A⁺S- fluorescent recipient (DK8615; Δ*pilQ*). Cells were first grown in CTT, harvested, resuspended to a calculated Klett of 250 and mixed. Strain mixtures were spotted on ½ CTT, 1% agarose pads (0.8 ml) prepared on glass slides and incubated at 33°C in a humid chamber. At various times cells were microscopically observed (20× objective). mCherry transfer was thus detected as emerging fluorescent flares from the inoculation spot, as donor cells were non-motile.

The second assay for mCherry transfer was as follows. Donors again expressed one of the mCherry reporters and recipients expressed cytoplasmic GFP that does not transfer (Wall and Kaiser, 1998). GFP allowed convenient identification of recipients. As described above donor and recipient strains were prepared. Alternatively, for liquid assays strains were prepared as described and transferred into a tube with 0.15 ml ½ CTT medium and incubated at 33°C without shaking. At various times cells were either scraped from agar plates or aliquoted from tubes, washed in TPM and pipetted onto a polylysine coated glass slides with a coverslip. Cells were observed with a Nikon Eclipse E800 microscope equipped with an array of objectives including a 100× 1.3 NA phase contrast oil submersion lens. Appropriate filter sets were used to visualize mCherry or GFP fluorescence. Images were captured with a Hamamatsu CCD camera and processed with Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA). To determine the per cent of recipients (GFP⁺) that received mCherry at least 50 and up to 200 cells were examined per sample or time point.

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References


