Biological and genetic factors regulating natural competence in a bacterial plant pathogen

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For naturally competent bacteria, spatially structured growth can provide an environment for enhanced horizontal gene transfer through transformation and recombination. DNA is often present in the extracellular environment, such as in the extracellular matrix of biofilms, and the lysis of a single cell can result in high local DNA concentrations. *Xylella fastidiosa* is a naturally competent plant pathogen that typically lives in a surface-attached state, yet previous work characterizing the competence of this organism was conducted with planktonic cells in liquid environments. Here, we show that transformation and recombination efficiencies are two to three orders of magnitude higher for cells grown on solid compared with liquid media, with maximum recombination efficiencies of about 10^{-3}. Cells were highly competent throughout their exponential growth phase, with no significant change in recombination efficiencies until population growth rates began to slow. Mutations in type IV pili, competency-related, and cell–cell signalling genes significantly impacted the ability of *X. fastidiosa* to acquire and incorporate DNA. Because *X. fastidiosa* is highly competent when growing in a surface-attached state, as it does within its insect vectors and host plants, recombination of naturally transformed DNA could be a significant route by which horizontal gene transfer occurs in natural environments.

INTRODUCTION

In natural environments, bacteria often exist as spatially structured populations, often adhered to a surface or interface (Costerton *et al.*, 1995). These environments often have high concentrations of extracellular DNA as a result of cell lysis, although some bacteria actively secrete DNA through type IV secretion systems or membrane vesicles (Hamilton & Dillard, 2006; Kadurugamuwa & Beveridge, 1995). It has been estimated that the lysis of a single cell can increase local DNA concentrations to above $100 \, \mu\text{g} \, \text{ml}^{-1}$ (Baur *et al.*, 1996). These high DNA concentrations and high cell densities make surface-attached populations, such as biofilms, ideal environments for horizontal gene transfer to occur through natural transformation. In fact, it has been shown that natural transformation occurs at rates 10- to 600-fold higher in *Streptococcus mutans* biofilms compared to planktonic cells (Li *et al.*, 2001).

Horizontal gene transfer can greatly affect the evolution, diversity and adaptation of bacteria. For naturally competent bacteria, transformation is one method of acquiring novel sequences. Nearly all naturally transformable bacteria use a series of competence-related proteins (encoded by *com* genes) for DNA uptake. In addition, many transformation systems include type IV pili or similar proteins (Burton & Dubnau, 2010; Chen & Dubnau, 2004; Hamilton & Dillard, 2006). Type IV pili may help stabilize the spatial structure of bacterial populations (Flemming & Wingender, 2010) and have been implicated in biofilm formation (O'Toole & Kolter, 1998). Several *pil* genes are involved in the function and structure of these type IV pili. The *pilQ* gene encodes a secretin that forms the outer membrane export pore for pili, and has been shown to bind DNA in the naturally competent *Neisseria meningitidis* (Assalkhou *et al.*, 2007; Chen & Dubnau, 2004). The *pilO* gene likely encodes a cytoplasmic membrane-anchoring domain, and PilB is a nucleotide-binding protein necessary for the assembly of pili (Mattick, 2002; Wall & Kaiser, 1999). A correlation has been shown between competence and the presence of pili in general and several *pil* genes in particular, but it is unclear if pili play a direct role in transporting DNA across the membrane (Chen & Dubnau, 2004). Once transforming DNA crosses the outer membrane, its transport through the periplasm and inner membrane is suggested to be mediated by a series of competence-related proteins (Chen & Dubnau, 2004; Hamilton & Dillard, 2006). Two important *com* genes are *comA*, which encodes a membrane channel that allows passage of DNA from the periplasm into the cytoplasm, and *comF*, which encodes the ATPase that helps drive DNA translocation (Chen & Dubnau, 2004; Tribble *et al.*, 2012).

Once inside the cytoplasm, DNA can be recombined into the genome or degraded for nutritional purposes. It has been hypothesized that competence originally developed as a nutrient uptake system (Redfield, 1993). However, if DNA is recombined into the genome, it can help generate or maintain genetic diversity. There is evidence that horizontally acquired sequences have increased virulence of existing pathogens and contributed to the emergence of new ones (Coupat-Goutaland et al., 2011; Friesen et al., 2006; Ma et al., 2006). Although potentially detrimental in stable environments, natural transformation in particular has been shown to increase the rate of adaptation of a bacterial pathogen to new environments (Baltrus et al., 2008).

Xylella fastidiosa is a vector-transmitted plant pathogen that was recently found to be naturally competent and able to undergo homologous recombination with acquired DNA (Kung & Almeida, 2011). X. fastidiosa is often found in natural environments as a plant endophyte, but causes disease when it multiplies to high populations inside the xylem vessels of susceptible hosts. There, cells inhibit the flow of xylem sap, leading to symptoms such as leaf scorching and stunted growth. X. fastidiosa is the causal agent of several important diseases of agricultural plants, such as Pierce's disease of grapevines, citrus variegated chlorosis and coffee leaf scorch (Chatterjee et al., 2008a). Host range and pathogenicity of different strains and subspecies of X. fastidiosa vary greatly. The genomes of representatives of the different X. fastidiosa subspecies contain a highly conserved set of core genes, likely responsible for adapting to life in xylem vessels and the foregut of insect vectors, along with a smaller set of flexible genes that likely varies between strains and subspecies (Nunes et al., 2003). Recent studies of this species have shown that homologous recombination plays a significant role in generating genetic diversity (Almeida et al., 2008; Scally et al., 2005; Yuan et al., 2010). In addition, some analyses suggest that recently emerged pathogenic strains of X. fastidiosa contain genetic elements potentially acquired from strains introduced from different geographical regions or infecting different host plants (Almeida et al., 2008; Nunes et al., 2003; Nunney et al., 2012).

In natural environments, X. fastidiosa primarily lives in biofilms, attached to either the xylem vessels of its host plants or the foregut of its insect vectors (Chatterjee et al., 2008a). Because of the linkage between spatially structured, surface-attached growth and horizontal gene transfer, we assessed the effects of these environments on the transformation and recombination efficiencies of this naturally competent pathogen, as all previous studies were conducted with planktonic cells (Kung & Almeida, 2011; Kung et al., 2013). We also determined the extent to which several com, pil and other genes contribute to X. fastidiosa's ability to acquire and recombine DNA.

METHODS

Strains, plasmids, media and growth conditions. X. fastidiosa subspecies fastidiosa strain Temecula (Van Sluys et al., 2003) was used in this study. Strain NS1-CmR, in which a chloramphenicol resistance cassette was introduced into a non-coding region of the genome, was obtained by transforming Temecula with pAX1-Cm (Matsumoto et al., 2009). A pglA mutant (strain Fetzer), in which the polygalacturonase gene has been disrupted by a kanamycin resistance cassette, was obtained from Roper et al. (Roper et al., 2007). All other mutants, which are kanamycin-resistant and constructed in the Temecula strain background, were either generated in this study or obtained from other sources (Table 1). All plasmids used in this study were suicide vectors that are unable to replicate independently in X. fastidiosa. Thus, antibiotic resistance was only conferred through chromosomal integration of the cassette. Plasmids p1k (Kung et al., 2013) and pAX1-Cm were used in recombination experiments. Plasmids for creating novel mutants were generated as described below. All plasmids were propagated in Escherichia coli strain EAM1, which expresses a X. fastidiosa methylase that increases the recombination efficiency of plasmids (Kung & Almeida, 2011; Matsumoto & Igo, 2010). Expression of the methylase was induced by supplementing the growth medium with 1 mM IPTG. X. fastidiosa cells were propagated on periwinkle wilt medium with (PWG) or without (PW) Gelrite (Hill & Purcell, 1995), or on X. fastidiosa minimal medium (XFM). We used liquid XFM as previously described (Kung & Almeida, 2011), and added 10 g l^{-1} of Gelrite for solidified medium. When appropriate, kanamycin was added to a final concentration of 30 μg ml^{-1} and chloramphenicol was added to a final concentration of 10 μg ml^{-1}. Cells were incubated at 28 °C with shaking at 180 r.p.m. when appropriate.

General transformation protocol. Transformation in liquid culture was done as previously described (Kung & Almeida, 2011). Cells were harvested from either solid PWG or XFM plates after approximately 1 week of growth and diluted in 200 μl liquid XFM to a final OD$_{600}$ of 0.01. After 2 days of growth at 28 °C with shaking, DNA was added to a final concentration of 5 μg ml^{-1}. Cells were plated on selective media after an additional 24 h of incubation. For transformation on solid plates, X. fastidiosa cells were harvested from PWG after approximately 1 week of growth and diluted to OD$_{600}$ of 0.25. Ten microlitres of cell suspension was spotted on approximately 5 ml of solid media (either PWG or XFM). Spots were allowed to dry and plates were incubated at 28 °C. At the appropriate time as determined by the experimental designs below, 1 μg of plasmids in approximately 10 μl was spotted directly on top of the cells. After a 24 h incubation period, cells were suspended in 250 μl buffer (50 mM NaCl and 10 mM Tris, pH 7.4) and dilution-plated on selective media. Antibiotic-resistant colonies were counted after approximately 2 weeks. Random samples of putative transformants were tested by PCR to confirm the correct insertion of the antibiotic marker at the desired locus using primers rpfF-fwd and rpfF-rev for p1k and primers NS1-f and NS1-r for pAX1-Cm (Kung & Almeida, 2011). A portion of the culture from each transformation experiment was also saved for quantification by quantitative polymerase chain reaction (qPCR), as previously described, to determine recombination efficiency, defined as the number of recombinants divided by the number of cells present (Kung & Almeida, 2011).

Media and inoculum effects on recombination efficiency. To compare recombination efficiencies on solid PWG (undefined, rich medium) and XFM (defined nutrient medium) plates, p1k was spotted on top of cells grown on solid plates after 2 days of growth. After an additional 24 h incubation period, cells were suspended and plated on PWG with kanamycin. This experiment was repeated, each time with 12 replicates per treatment.

To test the effect of the inoculum culture media on recombination efficiencies, cells were harvested from either solid PWG or XFM plates, inoculated into liquid XFM, and recombination efficiencies measured using the transformation procedure in liquid media with

Table 1. Genes tested for effects on recombination efficiency

NA, not applicable.

Locus	Name	Putative function	Pfam analysis	Mutant source
com genes				
0042	comF	Competence protein F	Phosporibosyltransferase domain	This study
0358	comA	DNA uptake protein	Competence protein family, metallo-β-lactamase domain, unknown function domain	This study
pil genes				
0023	pilY1	Type IV pilin biogenesis factor	*Neisseria* PilC domain	Li *et al.* (2007)
0062	fimA*	Type I pilus protein, surface attachment and biofilm formation	NA	Meng *et al.* (2005)
1691	pilQ†	Type IV fimbrial biogenesis outer membrane protein	STN family, secretin N domain, secretin family	Meng *et al.* (2005)
1693	pilO†	Type IV fimbrial biogenesis cytoplasmic membrane protein	PilO family	Li *et al.* (2007)
1927	pilB†	Type IV fimbrial biogenesis cytoplasmic ATPase	TS2E Nter domain, type II/IV SS protein family	Meng *et al.* (2005)
Cell–cell signalling				
0406	rpfC	Autoregulator of DSF biosynthesis	Response regulator domain, histidine kinase domain	Chatterjee *et al.* (2008b)
0407	rpfF	DSF biosynthesis	Enoyl-CoA hydratase/isomerase family	Newman *et al.* (2004)
Other genes				
0952	traD	Conjugal transfer protein	NA	This study
1017	ssb	ssDNA-binding protein	Single-stranded DNA-binding family domain	This study
1792	hxfB	Haemagglutinin adhesin	Haemagglutinin repeats, haemagglutinin activity domain, extended signal peptide of type V secretion system	Guilhabert & Kirkpatrick (2005)
1826	chiA	Chitinase	Glycosyl hydrolase family	F. Labroussaa & R. Almeida (unpublished)
1964	tolC	Multidrug resistance efflux pump, outer membrane export factor	Outer membrane efflux protein	Reddy *et al.* (2007)

*Mutant lacks type I pili.
†Mutant lacks type IV pili.

p1k with 18 replicates per treatment. Cultures were then plated on PWG with kanamycin. Antibiotic-resistant colonies from both experiments were counted and verified as described above.

Co-culturing strains on solid media. Strain NS1-CmR (chloramphenicol-resistant) and the *pglA* mutant (kanamycin-resistant, Fetzer strain background) were co-cultured on solid XFM to determine rates of recombination between strains growing on solid media. Cells were harvested from PWG after 1 week of growth and diluted to an OD_{600} of 0.25. Droplets of 10 µl of each strain were spotted directly on top of each other. Spots were allowed to dry for approximately 1 h before the second strain was added. Plates were incubated at 28 °C for 3 days before cells were resuspended and plated on PWG with kanamycin and chloramphenicol. An aliquot of each culture was saved for quantification by qPCR. After growth on selective media, a random sample of isolated doubly antibiotic-resistant colonies were screened with PCR using primers NS1-f/r and pglA-fwd/rev (Kung & Almeida, 2011) to confirm the presence of both antibiotic cassettes in the correct loci.

Population growth experiment. To study the effects of population growth on the competence of surface-attached cells, 10 µl samples of *X. fastidiosa* cell suspensions at an OD_{600} of 0.25 were spotted on solid XFM plates and incubated at 28 °C. To measure growth, four separate samples were harvested after 1, 2, 3, 4, 5, 7, 9, 11, 14 and 21 days of

growth by resuspending cells in 250 µl buffer, with an aliquot frozen for qPCR and another used for live/dead staining (see below). To measure recombination efficiency, six additional separate samples were transformed as described above after 1, 2, 3, 4, 5, 7, 9, 11, 14 and 21 days of growth by adding 1 µg of p1k in a volume of approximately 10 µl directly on top of the cells, incubating for an additional 24 h, and dilution-plating on PWG with kanamycin. Aliquots were also saved for qPCR and live/dead staining. Total cell numbers were determined by qPCR, and live cell numbers were determined by combining data from qPCR with the live/dead staining.

Resuspended cells from the growth experiment were stained with SYTO 9 (Invitrogen) and propidium iodide to estimate the ratio of live and dead cells. We added SYTO 9 and propidium iodide to final concentrations of 5 µM and 30 µM, respectively, in 50 µl of cell suspension and incubated the mixture for approximately 15 min in the dark at room temperature. Fluorescence was viewed with a Zeiss AxioImager M1 with Chroma filter 49002 (excitation filter BP450–490, emission filter BP500–550) for SYTO 9 staining and Chroma filter 31002 (excitation filter BP528–552, emission filter BP578–633) for propidium iodide staining. Portions of each slide were randomly selected for imaging and the ratio of live/dead cells was counted manually.

Generation of novel mutants. Mutants were used to determine whether or not certain genes are involved in competence and

recombination in *X. fastidiosa*. Four novel mutants were generated in this study. All PCRs for cloning purposes utilized Finnzymes Phusion Hot Start Polymerase (Thermo Fisher Scientific). Regions of between 300 and 1300 bp upstream and downstream of each target gene were amplified using primers comA-, comF-, traD-, ssb-fwd1/rev1 and fwd2/rev2 (Table 2) to create comA-, comF-, traD- and ssb-F1 and F2, respectively. Primers rev1 and fwd2 contained a *Sal*I restriction site, while fwd1 primers contained an *Nco*I site and rev2 primers contained a *Sac*I site. The F1 and F2 fragments for each gene were then digested with *Sal*I and ligated to form fragment F1F2, which was PCR-amplified using primers fwd1 and rev2. The F1F2 fragments were digested with *Nco*I and *Sac*I and ligated into vector backbone pGEM-5zf(+) (Promega) to create pF1F2. The kanamycin cassette from pAX1-Km (Matsumoto *et al.*, 2009) was amplified using primers KmF *Sal*I and KmR *Sal*I (Table 2). The kanamycin cassette and pF1F2 were digested with *Sal*I and ligated to form p*comA*::kanR, p*comF*::kanR, p*traD*::kanR and p*ssb*::kanR. These plasmids were propagated in *E. coli* strain EAM1 and used to transform *X. fastidiosa* strain Temecula using either the liquid or solid transformation procedure, with cells growing for 2 days prior to addition of DNA. Recovered antibiotic-resistant colonies were screened by PCR and sequenced to confirm the replacement of the majority of the coding region of each gene with the kanamycin cassette.

Transformation of mutant strains. Mutant strains (Table 1) were transformed on solid XFM plates using pAX1-Cm (Matsumoto *et al.*, 2009). Six samples of each mutant were transformed on a given day, and recombination efficiencies were measured on two to six different days for each mutant. A value of 1×10^{-30} was added to each recombination efficiency so efficiencies could be log-transformed for statistical analysis to fit model assumptions of homogeneous variance. Log-transformed efficiencies were subjected to an ANOVA using a generalized linear model with mutant as a fixed factor and day as a random factor in the statistical package R (v. 2.15.2, R Foundation for Statistical Computing) using the 'lmer' function in package 'lme4' (Bates & Maechler, 2013). Differences between mutants were determined by Tukey's HSD test using the 'glht' function in package 'multcomp' (Hothorn *et al.*, 2008). Antibiotic-resistant colonies for each mutant were randomly selected for screening by PCR using primers NS1-f and NS1-r (Kung & Almeida, 2011) to confirm insertion of the chloramphenicol cassette in the NS1 region.

RESULTS

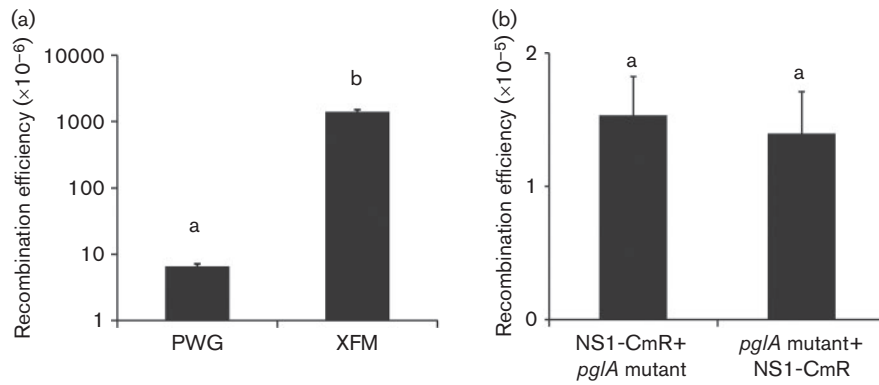### Recombination on solid media is highly efficient

When cells were transformed on solid media with p1k (Kung *et al.*, 2013), a plasmid containing a kanamycin cassette flanked on either side by approximately 1 kb of homologous *X. fastidiosa* DNA, recombination rates of 1.4×10^{-3} and 6.5×10^{-6} were observed for cells grown on XFM and PWG respectively (Fig. 1a). Data from two separate experiments were combined after determining that results were normally distributed and a *t*-test revealed no significant difference between the two experiments ($t_{18df}=0.79$, $P=0.440$ and $t_{14df}=2.11$, $P=0.54$ for PWG and XFM respectively). Previous studies showed recombination efficiencies of 5.62×10^{-5} for the same plasmid in liquid XFM (Kung *et al.*, 2013) and that natural competence was undetectable for cells grown in liquid PW (Kung & Almeida, 2011). Recombination efficiency on solid XFM was significantly higher than on solid PWG ($t_{23df}=11.98$, $P=2.29 \times 10^{-11}$). Recombination of the antibiotic markers between strain NS1-CmR (chloramphenicol-resistant) and the *pglA* mutant (kanamycin-resistant) when cells were co-cultured on solid XFM was readily detected (Fig. 1b), with doubly antibiotic-resistant colonies recovered in approximately one out of every 10^5 cells present. All PCR-screened colonies were positive for recombination. Previous work in liquid XFM resulted in recombination in approximately one out of every 10^7 cells present (Kung & Almeida, 2011). The order in which the strains were spotted on the plate did not affect recombination efficiencies ($t_{10df}=0.32$, $P=0.76$).

Phenotype of inoculum cells minimally affects recombination efficiency in liquid medium

Previous results indicated that recombination efficiency of planktonic cells initially harvested from solid media decreased over time (Kung & Almeida, 2011). To determine if this decrease was an effect of cells transitioning from a solid-state to planktonic growth phenotype, recombination efficiencies in liquid XFM were measured for cultures inoculated with cells harvested from solid PWG (undefined, nutrient-rich medium) or XFM (defined nutrient medium) (Fig. 2). As cells were much more competent when transformed on solid XFM compared to PWG, we would expect the difference in recombination efficiencies to carry over to liquid media if the decrease over time was caused by the transition to planktonic growth. Previous work has shown that during the time-course of this experiment, cells enter exponential growth in liquid XFM

Table 2. Primers used in this study

Function	Sequence 5′–3′
comA mutant	
fwd1	ATACCATGGGCAACGTATGTCGG
rev1	AATGTCGACAACAGCGAGCATTACT
fwd2	TTAGTCGACATGCTGGAACTGGTAAAG
rev2	TTAGAGCTCAAGTAAAGCACCACATGC
comF mutant	
fwd1	AACCCATGGCGATGATTTATTGAG
rev1	ATAGTCGACCTACATGCAGCAGCA
fwd2	GTAGTCGACCAGACTGCCGAACTT
rev2	ATAGAGCTCAACGTCCATCAACTTCTG
ssb mutant	
fwd1	TTTCCATGGGCTATACCGCTACC
rev1	CACGTCGACTAGGATCACCTTGTTAA
fwd2	ATTGTCGACTTCCACGATGACG
rev2	TAAGAGCTCGCACCTGCTTTGAT
traD mutant	
fwd1	CCTCCATGGTATGTCAGCATC
rev1	GGTGTCGACTTCACCAGAGTC
fwd2	TATGTCGACGTTCATCGCGTC
rev2	TATGAGCTCTGGAAGCAACAA
Kanamycin cassette	
KmF *Sal*I	ATAGTCGACCTCAACCATCATCGA
KmR *Sal*I	CAGGTCGACTCTAGAGGATCCC

Fig. 1. Recombination efficiency of cells on solid media. (a) Recombination efficiencies of strain Temecula transformed with p1k. Rates of 6.5×10^{-6} were observed for cells grown on PWG and 1.4×10^{-3} for cells grown on XFM ($P = 2.29 \times 10^{-11}$, $n = 24$). (b) Efficiency of marker gene transfer between different strains of *X. fastidiosa* spotted on top of each other on solid XFM. The *x*-axis indicates the order in which strains were spotted on plates. The order in which the cells were spotted on the plate did not significantly affect recombination efficiencies ($P = 0.76$, $n = 12$). Letters indicate statistically different treatments.

(Kung & Almeida, 2011) and our qPCR data showed final cell numbers were significantly higher than the initial inoculum, so the population should have transitioned to planktonic growth. Following the protocol for transformation in liquid media, we calculated recombination rates of 1.57×10^{-6} for cells initially grown on solid PWG and 5.58×10^{-6} for cells initially grown on solid XFM. The differences were not on the same order of magnitude as what was observed on solid media, but were still statistically significant ($t_{22df} = 4.18$, $P = 0.0004$).
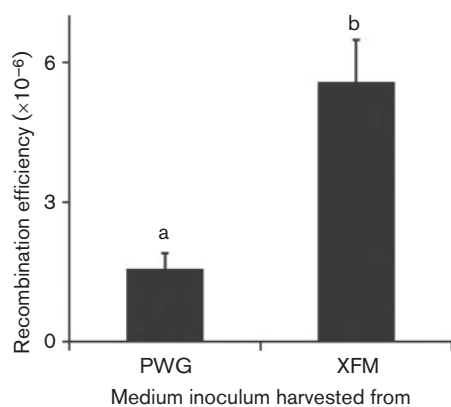

Fig. 2. Effect of initial inoculum growth medium on competence in liquid XFM. Cells were grown for approximately 1 week on solid PWG or XFM before being diluted to OD_{600} of 0.01 in liquid XFM. The average recombination efficiency for cells initially grown on PWG was 1.57×10^{-6}, and 5.58×10^{-6} for cells initially grown on XFM ($P = 0.0004$, $n = 18$). Different letters indicate statistically different treatments.

Competence is highest during exponential growth and is correlated with cell population rate of change

To determine if recombination efficiencies of cells growing in a surface-attached, spatially structured state decreased over time, we measured growth and recombination efficiencies for cells treated with p1k on solid XFM over the course of 21 days (Fig. 3a). Cells grew exponentially between 1 and 5 days after inoculation ($r^2 = 0.86$, $P < 1 \times 10^{-11}$) with a doubling time of 1.53 days ($P = 2.6 \times 10^{-9}$). During this time, 85–95 % of the cells were viable, as measured with a live/dead stain. Maximum total cell numbers were reached at 7 days post-inoculation, with the proportion of live cells decreasing after that time. By 9 days post-inoculation, only 74 % of cells were still viable, and viability continued to decrease, with only 28 % of cells still viable at day 21.

When cells were exposed to p1k between 1 and 5 days after inoculation, the recombination efficiency (number of recoverable antibiotic-resistant colonies divided by the number of viable cells) was about 10^{-4}, with no significant difference between days as determined by Tukey's HSD test ($P > 0.05$). Recombination efficiencies decreased by an order of magnitude to 2.9×10^{-5} when cells were transformed at day 7 and continued to decrease over the remainder of the experiment to an efficiency of 9.9×10^{-9} when cells were transformed after 21 days of growth. Recombination efficiencies for cells grown for 5 days or less prior to exposure to p1k were significantly higher than for cells exposed to p1k after 7 days or more of growth. A regression analysis found a significant correlation between the negative log-transformed recombination efficiencies and the rate of change of the cell population between days 2 and 14 ($y = 0.0632 \times -8.38$, $r^2 = 0.914$, $P = 3.97 \times 10^{-26}$), indicating that competence was positively correlated with the rate of change of the cell population (Fig. 3b).
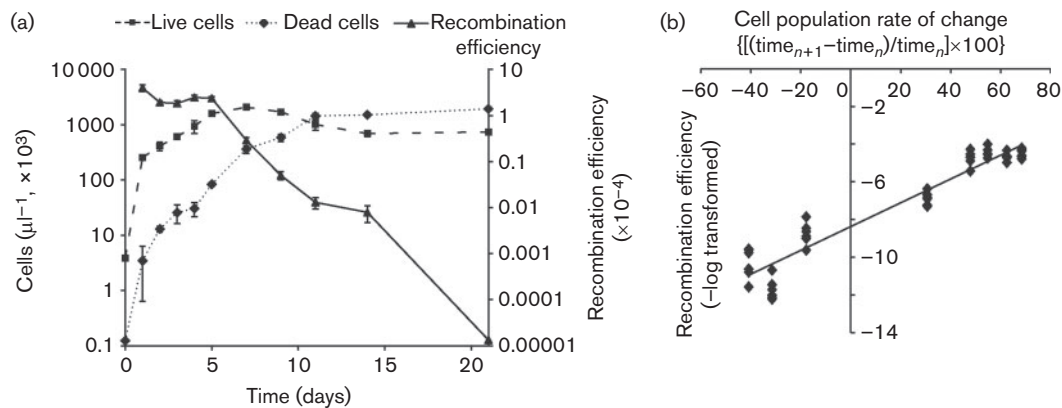
Fig. 3. Competence and growth of *X. fastidiosa* on solid XFM over time. (a) Live (squares, dashed line) and dead cell populations (diamonds, dotted line) were quantified using qPCR and SYTO 9/propidium iodide staining to determine the ratio of live/dead cells over the course of 21 days. Recombination efficiencies of live cells (triangles and solid line, secondary axis) remained relatively constant during exponential growth and then dropped by several orders of magnitude as the population as a whole began to die. (b) A correlation was found between the negative log-transformed recombination efficiencies of live cells and the rate of change of the population of live cells ($y=0.0632\times-8.38$, $r^2=0.914$, $P=3.97\times10^{-26}$). Data from day 1 and day 21 were outliers and removed from the analysis.

Disruption of genes encoding type IV pili, outer membrane pumps, regulators and *com* genes affect transformation and recombination efficiencies

Transformation and recombination efficiencies for wild-type Temecula and 14 mutant strains (Table 1), including four novel mutants, were measured to determine which genes affect competence in *X. fastidiosa*. Candidate genes for generating novel mutants were selected based on either homology to known competence-related genes in other organisms (*comA* and *comF*) (Chen & Dubnau, 2004; Hamilton & Dillard, 2006) or previous microarray data indicating upregulation when grown in liquid XFM compared with liquid PW (*traD*, putative conjugal transfer protein, and *ssb*, single-stranded DNA-binding protein) (Killiny & Almeida, 2009), as cells are more competent in XFM than PW (Kung & Almeida, 2011). Type IV pili and a series of *com* genes are necessary for efficient transformation in a range of naturally competent bacteria (Burton & Dubnau, 2010; Chen & Dubnau, 2004; Hamilton & Dillard, 2006), and previous work has suggested that cell–cell signalling may affect competence in *X. fastidiosa* (Kung & Almeida, 2011). A variety of other strains with mutations in genes not expected to affect competence were selected as controls (*fimA*, *chiA*, *hxfB*, *tolC*). The *fimA*, *rpfC*, *ssb*, *hxfB* and *chiA* mutants exhibited recombination efficiencies that were statistically equivalent to the wild-type ($P>0.05$, Fig. 4). The *traD*, *comF*, *pilY1*, *pilB*, *rpfF* and *tolC* mutants had significantly lower recombination efficiencies, and no recombination was detected for the *comA*, *pilQ* or *pilO* mutants. All PCR-screened recovered antibiotic-resistant colonies were positive for recombination.

DISCUSSION

Xylella fastidiosa naturally and stably acquired extracellular DNA at efficiencies approximately 100 times higher when grown on solid media compared to liquid media. When exogenous plasmid was added, very high rates of transformation were observed (up to 10^{-3}) on solid XFM, while transformation efficiency of the same plasmid in liquid XFM is only about 10^{-5} (Kung *et al.*, 2013). In addition, transformation rates of approximately 10^{-6} were observed on solid PWG, while competence of cells grown in liquid PW was previously undetectable (Kung & Almeida, 2011).

We observed similarly high recombination efficiencies between different antibiotic-resistant strains of *X. fastidiosa* when co-cultured on solid XFM. Marker gene transfer occurred at a frequency of about 10^{-5}, while recombination rates of 10^{-7} were observed in previous studies in liquid XFM (Kung & Almeida, 2011). As in the previous study, actual recombination rates were likely higher than observed, as any recombination events not involving the transfer of marker genes would not have been detected. These results also indicate that extracellular DNA provided by neighbouring cells is sufficient to transform competent cells in the population, as shown in other bacteria such as *Vibrio cholerae* biofilms (Meibom *et al.*, 2005).

As population growth was likely the dominant factor linked to decreased recombination in broth culture (Kung & Almeida, 2011), we assessed growth effects on surface-grown cells. Overall, competence was linked directly with the rate of population growth. Recombination efficiencies remained relatively constant during exponential growth,
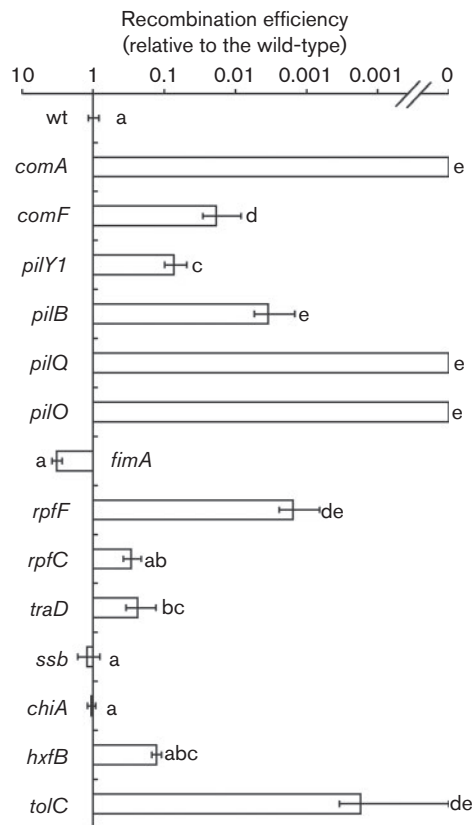
Fig. 4. Relative average recombination efficiencies of *X. fastidiosa* mutants on solid XFM compared to the wild-type (wt). Letters indicate statistically different groups (*P*<0.05). All *com* and type IV *pili* gene mutants tested had significantly lower recombination efficiencies than the wild-type, as did the *rpfF*, *tolC* and *traD* mutants. The *fimA*, *rpfC*, *ssb*, *chiA* and *hxfB* mutants all had transformation and recombination efficiencies statistically equivalent to the wild-type. Error bars indicate SE.

then dropped by several orders of magnitude as the population entered stationary and death phase. In *Ralstonia solanacearum*, to our knowledge the only other documented naturally competent plant pathogen, there is a similar correlation between competence and exponential growth (Bertolla *et al.*, 1999). Similar patterns of growth-phase dependent competence are also seen in *Acinetobacter* sp. strain BD413 (Palmen *et al.*, 1993). However, in *Bacillus subtilis*, for example, the onset of competence occurs during late exponential growth or early stationary phase (Grossman, 1995), and growth rate deceleration is one of the inducers of competence in *V. cholerae* (Meibom *et al.*, 2005).

Competence was highest when *X. fastidiosa* cells were growing exponentially and the majority (>95 %) of the cells were alive. This could limit the amount of donor DNA available in natural environments since transforming DNA would likely be supplied by neighbouring (dead) cells rather than by the addition of exogenous DNA as done

here. However, we observed high recombination rates (10^{-5}) when two different strains were co-cultured together on plates, and the lysis of a single cell can result in local concentrations of DNA of up to 100 μg ml^{-1} (Baur *et al.*, 1996). In addition, in *R. solanacearum*, it has been hypothesized that cells lysed during the initial infection process can provide sufficient DNA for transformation as cells begin to grow exponentially inside the host plant (Bertolla *et al.*, 1999). Perhaps *X. fastidiosa* cells are also mechanically lysed during the vector transmission process. Alternatively, *X. fastidiosa* may actively export DNA as observed in other taxa, such as *Neisseria gonorrhoeae* (Hamilton & Dillard, 2006), although no evidence has been found in support of this.

We also attempted to transform a variety of mutants grown on solid medium to determine the effects of certain genes on DNA acquisition in *X. fastidiosa*. Studies conducted with other naturally competent organisms indicated that several of the genes tested here are required for efficient natural transformation (Carruthers *et al.*, 2012; Chen & Dubnau, 2004; Hamilton & Dillard, 2006; Meier *et al.*, 2002). Performing the experiments on solid media enabled detection of low-probability events in certain mutant backgrounds. Recombination efficiencies also remained stable for longer periods of time on solid media. As expected, disruption of several genes not presumed to be involved in DNA uptake (type I pilus protein *fimA*, chitinase *chiA* and haemagglutinin adhesion *hxfB*) had no effect on competence. Disruption of *ssb*, which encodes a single-stranded DNA-binding protein, did not significantly impact DNA transformation and recombination rates. However, a BLAST search revealed that multiple copies of this gene are present in the genome. The *tolC* mutant, however, was significantly less transformable than the wild-type. TolC is one of three component proteins composing the multidrug resistance efflux pump which traverses the periplasm and outer membrane (Reddy *et al.*, 2007). It is possible that a loss of TolC could affect DNA uptake across the outer membrane, even if the protein is not directly involved in DNA transport. The mutant for the putative conjugal transfer gene *traD* had slightly lower recombination efficiencies than the wild-type. Even though conjugation and transformation are distinct processes, DNA transport across the membrane may also be impacted in this mutant.

Transformation and recombination efficiencies were lower in all four type IV pili mutants tested. Two of the type IV pili mutants tested were transformable, but at efficiencies at least 10-fold lower than the wild-type. Disrupting *pilY1* had the least pronounced effect on competence, with efficiencies about 7 % of the wild-type. The product of the *pilY1* gene is thought to be involved in cell adhesion and localized at the pilus tip (Johnson *et al.*, 2011; Li *et al.*, 2007). In *X. fastidiosa*, *pilY1* mutants still produce type IV pili that appear similar to wild-type pili, but the mutants have reduced twitching motility (Li *et al.*, 2007). Thus, the necessary structure for DNA transport through the membranes in *pilY1* mutants may still be intact. For the

other three *pil* mutants tested either recombination efficiency was markedly lower (approx. 0.35 % of wild-type for the *pilB* mutant) or transformation was undetectable (*pilO* and *pilQ* mutants). Previous work has shown that these three *pil* mutants do not produce any type IV pili (Li *et al.*, 2007; Meng *et al.*, 2005). As the PilB, PilO and PilQ proteins in *X. fastidiosa* are likely required for pilus assembly and retraction, a mutation in any of the genes encoding these proteins is much more likely to lead to non-functional pili compared to a mutation in *pilY1*. Disruption of putative *com* genes also led to decreased (*comF*) or no observable (*comA*) recombination. Thus, DNA transport from the extracellular environment into the cytoplasm in *X. fastidiosa* likely occurs in a similar fashion to what has been observed in other bacteria, requiring a type IV pilus or like structure and a series of Com proteins (Busch *et al.*, 1999; Chen & Dubnau, 2004; Hamilton & Dillard, 2006; Meibom *et al.*, 2005; Meier *et al.*, 2002). While many of the *pil* genes in *X. fastidiosa* are grouped together in several operons (Li *et al.*, 2007; Meng *et al.*, 2005), the *com* genes tested here do not appear to be in operons with other competence-related genes.

Significantly lower transformation and recombination efficiencies were observed in an *rpfF* mutant (approx. 0.15 % of wild-type efficiencies), but an *rpfC* mutant recombined at the same rate as the wild-type. Cell–cell signalling in *X. fastidiosa* is mediated by the small molecule *D*iffusible *S*ignalling *F*actor (DSF); RpfF is a DSF synthase, while RpfC is a two-component regulator that senses DSF. In *X. fastidiosa*, *rpfF* mutants do not produce DSF, while *rpfC* mutants overproduce DSF (Newman *et al.*, 2004), and the two mutants essentially exhibit opposite phenotypes for traits controlled by genes regulated by RpfF and RpfC (Chatterjee *et al.*, 2008b). It is possible that *X. fastidiosa* relies on DSF to indicate the presence of other cells, and therefore a potential source of donor DNA, before becoming competent. Quorum sensing has been implicated in the induction of competence in other bacteria, such as *V. cholerae*, *B. subtilis* and several species of *Streptococcus* (Dubnau *et al.*, 1994; Håvarstein *et al.*, 1997; Suckow *et al.*, 2011). Surprisingly, however, maximum competence of *X. fastidiosa* wild-type cells was seen in relatively young cultures when the DSF concentration would have been lower than at later growth stages. It is possible that in *X. fastidiosa* DSF affects competence through its regulation of gene expression rather than by indicating cell populations. It is also interesting to note that in PW, *rpfF* mutants do not form biofilms as readily as the wild-type (Wang *et al.*, 2012).

Increased rates of transformation have been observed in naturally competent bacteria living in biofilms and other spatially structured environments (Hendrickx *et al.*, 2003; Li *et al.*, 2001; Madsen *et al.*, 2012; Maeda *et al.*, 2006). Extracellular DNA is often present at high concentrations in these environments, and there is evidence that it is required for efficient biofilm formation in *X. fastidiosa* (Cheng *et al.*, 2010). Our work has shown that transformation and recombination rates in *X. fastidiosa*

increased by over 100-fold in surface-attached cells compared to planktonic cells. As *X. fastidiosa* is naturally competent and primarily lives in a surface-attached state (Chatterjee *et al.*, 2008a; Kung & Almeida, 2011), natural transformation could provide an efficient route for horizontal gene transfer should two different strains co-colonize a single insect vector or host plant. Further studies documenting the extent to which such gene transfer occurs in natural environments could provide insight into the evolution and emergence of new pathogenic strains.

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