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Horizontal gene transfer through natural competence and recombination in the generalist plant pathogen *Xylella fastidiosa*

by

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Abstract

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Doctor of Philosophy in Microbiology

University of California, Berkeley

Professor Rodrigo P. P. Almeida, Chair

Horizontal gene transfer has been implicated as a contributing factor towards the diversity and adaptation of pathogens, and the emergence of new diseases. For naturally competent bacteria, DNA acquired through transformation and recombined into the genome could provide a means for this genetic transfer to occur. The work presented here illustrates that *Xylella fastidiosa*, a bacterial pathogen responsible for several important plant diseases, is naturally competent and able to homologously recombine acquired DNA into its genome. *Xylella fastidiosa* is vector-transmitted and often exists in natural environments as an endophyte, but causes disease when it multiplies to high levels inside the xylem vessels of its host plants, causing symptoms such as leaf scorching and stunted growth. Several factors were identified that affect the competence of *X. fastidiosa*, including nutrient availability, growth stage, and methylation state and size of transforming DNA. Recombination efficiencies for *X. fastidiosa* were at least 1000-fold higher when cells were grown in a defined nutrient medium compared to cells grown in a rich medium. In addition, surface-attached cells transformed and recombined DNA at efficiencies approximately two orders of magnitude higher than their planktonic counterparts. Maximum recombination efficiencies, defined as the number of recombinant cells recovered divided by the total number of cells, were approximately 10^{-3} when high concentrations of exogenous plasmid DNA were added to cells, and 10^{-5} when strains harboring different antibiotic markers were co-cultured on solid medium. For planktonic cells, maximum recombination efficiencies were only approximately 10^{-5} when DNA was added and 10^{-7} when different strains were co-cultured. Cells appeared most competent while undergoing exponential growth. For planktonic cells, competence peaked after two days of growth and then rapidly declined, with no recombination observed after 8 days. In biofilms, however, cells remained highly competent for at least five days, with recombination events observed even after 21 days of growth. The transformation mechanism in *X. fastidiosa* is likely similar to that of other naturally competent bacteria, with mutations in type IV pili, competence-related genes (*com* genes), and cell-cell signaling genes impacting competence.

It was also experimentally determined that flanking homologous sequences as short as 96bp in transforming DNA is sufficient to initiate recombination, with efficiencies increasing exponentially with length of the homologous region up to 1kb. In addition, recombination efficiencies decreased exponentially with the size of non-homologous insert. Integration of up to 4kb of non-homologous DNA was observed experimentally. An *in silico* analysis of genomic sequences confirmed that the experimental data was consistent with events detected in natural populations, with an estimated mean size of recombination events of 1,906 bp. Each recombination event also modified, on average, 1.79% of the nucleotides in the recombined region. Based on sequence similarity of shared coding regions, it appears that recombination between different subspecies of *X. fastidiosa* could frequently occur.

Originally, it was thought that *X. fastidiosa* was primarily clonal, but recent studies have suggested that recombination plays a significant role in generating genetic diversity in this bacterium. The work presented here illustrates that *X. fastidiosa* is naturally competent and that DNA acquired through natural transformation could be a substantial source of donor DNA for recombination. Understanding how this process is regulated and what factors affect its efficiency could provide insight into the genetic diversity of this organism and how new diseases emerge.

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Chapter 1: Introduction

Although the importance of new diseases threatening agriculture and public health has become evident in recent decades, limited research has addressed on the evolutionary forces that drive pathogen diversification that lead to disease emergence. The potential contribution of horizontal gene transfer and recombination has recently been brought to attention, however, especially for pathogen evolution (1–3). For naturally competent bacteria, which are able to acquire exogenous DNA from the environment under natural growth conditions, recombination of extracellular DNA can provide one means of horizontal gene transfer. I report that *Xylella fastidiosa*, a bacterial plant pathogen responsible for many important and emerging diseases, is naturally competent and able to homologously recombine acquired DNA when grown in medium designed to mimic xylem sap, its natural host plant environment. By studying this trait in an ecologically relevant pathogen, I determined what factors regulate this process, a likely mechanism by which it occurs, and potential implications of horizontal gene transfer to this pathogen's evolution.

Xylella fastidiosa is the etiological agent of many important diseases such as Pierce's disease of grapevine, citrus-variegated chlorosis, and coffee leaf scorch (4). In natural environments, *X. fastidiosa* often exists as an endophyte in the xylem of plants showing no symptoms, but causes disease when it multiplies to high populations inside the xylem vessels of its host plants, inhibiting the flow of xylem sap. Leafhopper vectors transmit *X. fastidiosa* between host plants, and the bacteria can attach to and multiply on the walls of the insects' foreguts. The subspecies and strains of *X. fastidiosa* differ in their ability to colonize, move within, or cause disease to different host plants (5). The genes and proteins responsible for host specificity have not been identified, although differential gene regulation can account for host specificity in at least one strain (6). Almost nothing is known about the underlying genetic mechanisms enabling *X. fastidiosa* emergence in diverse agroecosystems. Previous work hypothesized that this bacterium was primarily clonal, but recent research has suggested recombination between strains is common (7–10). Sharpshooter vectors can encounter a variety strains of *X. fastidiosa* while feeding on different plant hosts, possibly allowing mixed communities of *X. fastidiosa* to coexist inside the insects and facilitating horizontal gene transfer. In addition, some results suggest that recently emerged pathogenic strains contain genetic elements likely transferred between endemic and introduced strains or strains with different host ranges (11). However, factors affecting recombination and its larger implications for pathogen emergence in this system have not previously been described.

Horizontal gene transfer has been implicated in the emergence of new plant diseases (12, 13). Recombination of naturally transformed DNA in particular has been shown to increase the virulence of *Ralstonia solanacearum* isolates, another naturally competent plant pathogen that colonizes the xylem of its hosts, causing bacterial wilt (14). In addition, there is evidence that recombining pathogens are better able to adapt to new environments (15). Recombination generates and maintains diversity and can contribute to gene flow and cohesiveness within a population (2). Studies have also shown that genomic plasticity within a recombining pathogen population allows rapid adaptation and development of antibiotic resistance (16). Recombination may also be especially relevant for *X. fastidiosa*, as well as other generalist pathogens, as it may allow the population as a whole to maintain the allelic diversity necessary to explore a wide variety of potential hosts. This may explain why *X. fastidiosa* typically exists as a non-pathogenic endophyte in natural environments; it may sacrifice the ability to thrive in any

particular host for the ability to exploit a wide variety of hosts. However, once the bacterium encounters an agricultural environment in which the potential host plants are usually genetically similar (if not identical), a selective sweep can occur, with the most-fit genotype emerging. In fact, it has been shown that host-specialized pathogens tend to recombine at lower frequencies than their non-specialized relatives (17). It has been hypothesized that the emergence of new pathogens is characterized by high initial recombination rates as the pathogen adapts to its new environment, which drop after it has established itself in its new niche (18). Recombination may still be occurring in such populations but be undetectable (e.g. recombination between identical sequences). However, as I show that several pathogenic strains of *X. fastidiosa* are in fact naturally competent and able to recombine acquired DNA, it is unlikely that these pathogenic strains lose their ability to undergo horizontal gene transfer.

Understanding the dynamics affecting natural competence and recombination in *X. fastidiosa* is highly relevant both from a basic and applied science perspective. Horizontal gene transfer is a major contributor to genetic diversity and adaptation, and natural transformation is one of three major pathways through which it can occur. For *X. fastidiosa* in particular, understanding this process is important as several new diseases have recently emerged. In addition, the potential for different strains to come into contact with each other and exchange DNA has increased as a result of intensifying and changing agricultural practices, movement of strains across geographic regions, and the expansion of vector ranges. The following three chapters present my research illustrating that *X. fastidiosa* is naturally competent and able to homologously recombine acquired DNA into its genome, as well as the different factors that regulate competence, and potential implications of this process. As they have been prepared for independent publication, some redundancy among chapters was unavoidable.

Chapter 2, entitled "Natural competence and recombination in the generalist plant pathogen *Xylella fastidiosa*," documents my initial discovery of how *X. fastidiosa* is able to naturally acquire and homologously recombine extracellular DNA into its genome. Transformation and recombination occurred when high concentrations of exogenous DNA, either in circular or linear form, were supplied to planktonic cells grown in a defined nutrient medium. In addition, exchange of antibiotic markers occurred between co-cultured strains in defined nutrient medium. Several factors were identified that regulate natural competence, including nutrient availability, methylation state of transforming DNA, and population growth. In a liquid medium, cells were most competent as they entered exponential growth, and recombination efficiencies declined as the population continued to grow. No recombination was detectable after populations reached stationary phase. These experiments indicated that natural transformation can occur at relatively high frequencies in *X. fastidiosa*, suggesting that recombination of naturally transformed DNA is a likely route for horizontal gene transfer in this organism. This chapter was published by the American Society for Microbiology in *Applied and Environmental Microbiology* in August 2011.

Once I found that *X. fastidiosa* was naturally competent, I sought to determine how the size of potentially transforming DNA affects recombination efficiency. Large insertion events could potentially introduce novel genes or open reading frames, while smaller recombination events would likely result in the transfer of new alleles or single nucleotide polymorphisms. In Chapter 3, I experimentally determined how the size of homologous and non-homologous DNA affects recombination efficiencies and conducted an *in silico* analysis to sample the effects of recombination in one population of *X. fastidiosa*. I found that a maximum of 96 base pairs of DNA with homology to chromosomal loci must flank incoming DNA to initiate recombination,

and additive integration can recombine at least 4kb of non-homologous DNA. The *in silico* sequence analysis confirmed that these numbers reflect actual recombination events in one natural population of *X. fastidiosa*, and that recombination between different subspecies could occur relatively efficiently. In other organisms, the presence of flanking regions of identity can remove most recombinatorial barriers caused by sequence divergence (19). As the different subspecies of *X. fastidiosa* share a conserved gene pool (11), the observed minimum of 96 bp necessary to initiate recombination and the *in silico* analysis of a natural population suggest that recombination between even distantly related subspecies could occur. In addition, single genes can alter host range or pathogenicity (6, 20), so the insertion of 4kb of novel DNA could significantly impact the biology of any given strain. This chapter, entitled "Effects of DNA size on transformation and recombination efficiencies in *Xylella fastidiosa*," was published by the American Society for Microbiology's *Applied and Environmental Microbiology* in March 2013.

The final chapter of my dissertation delves into the relationship between horizontal gene transfer and biofilms. For naturally competent bacteria, biofilms can provide the ideal environment for transformation to occur as DNA is often an integral component of the extracellular matrix that binds the cells together. This potential relationship is also ecologically relevant as *X. fastidiosa* primarily exists as a biofilm in natural environments, attached either to the xylem vessels of its host plants or the foregut of its insect vectors. I found that surface-attached *X. fastidiosa* cells naturally acquired and recombined DNA at efficiencies approximately two orders of magnitude higher than planktonic cells. In addition, populations of cells in biofilms remained highly competent throughout the exponential growth phase, and recombination events were still detectable even after 21 days of growth. I also explored the mechanism of DNA transport into the cytoplasm, and tested the recombination abilities of a variety of *X. fastidiosa* mutants. I found that, similar to other documented naturally competent bacteria, mutations in type IV pili, competency related genes (*com* genes), and genes enabling cell-cell signaling were required for *X. fastidiosa* to efficiently transform and recombine DNA. These results suggest that horizontal gene transfer through natural transformation and recombination could occur at high frequencies should two strains of *X. fastidiosa* come in contact with each other, such as by colonizing the same host plant or insect vector. This chapter, entitled "Biological and genetic factors regulating natural competence in a bacterial plant pathogen," has been prepared for publication.

There are many possible follow up questions and future directions available for those wishing to expand on this work. Understanding if and where this process physically occurs (inside the host plant, insect vector, etc.) in natural environments could provide insight into possible ways to mitigate the emergence of new pathogenic strains. This knowledge could be especially relevant as insect vectors visit multiple plants and are likely to come in contact with multiple strains in areas where *X. fastidiosa* is endemic. The introduction of invasive vectors with expanded ranges could exacerbate this problem. In addition, if the virulence or host range of certain strains can be increased by enabling them to recombine with DNA from other strains, as seen in *R. solanacearum* (14), sequencing these new recombinant isolates could aid in our understanding of what genes regulate pathogenicity. Extensive sequencing of non-pathogenic, environmental isolates has not been done; such a study could help define the total genomic diversity of *X. fastidiosa* and thus the extent of recombination within the species. Such a comparative analysis could potentially shed light on what contributes to pathogenicity or host range. Tackling these questions would greatly contribute to both basic and applied research, as

broadly relevant evolutionary and genomic information could be gleaned from studies of this emerging agricultural pathogen.

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Chapter 2: Natural competence and recombination in the generalist plant pathogen *Xylella fastidiosa*

ABSTRACT

Homologous recombination is one of many forces contributing to the diversity, adaptation, and emergence of pathogens. For naturally competent bacteria, transformation is one possible route for the acquisition of novel genetic material. This study demonstrates that *Xylella fastidiosa*, a generalist bacterial plant pathogen responsible for many emerging plant diseases, is naturally competent and able to homologously recombine exogenous DNA into its genome. Several factors were identified that affect transformation and recombination efficiencies, such as nutrient availability, cell growth, and methylation of transforming DNA. Recombination was observed in at least one out of every 10^6 cells when exogenous plasmid DNA was supplied and one out of every 10^7 cells when different strains were grown together *in vitro*. Based on previous genomic studies and experimental data presented here, there is mounting evidence that recombination can occur at relatively high rates and could play a large role in shaping the genetic diversity of *X. fastidiosa*.

INTRODUCTION

Naturally competent bacteria, which are able to uptake DNA under natural growth conditions, are found in a wide range of phyla, suggesting that this trait is functionally important and could confer a fitness benefit (42). For these bacteria, DNA acquired through natural transformation could recombine into the genome, providing a source of genetic diversity and potentially a means of horizontal gene transfer, although it is possible that natural transformation evolved as a nutrient uptake system (37). Many factors affect the onset of competence in different bacteria. Bacteria can become competent in response to environmental signals or cues, such as antibiotics or alkaline conditions (10). Nutritional factors can also play a role. For example, the presence of chitin induces competence in *Vibrio cholerae* (32), while starvation conditions induce competence in *Haemophilus influenzae* (28). Growth stage can also be a regulating factor (10). A notable outlier, however, is *Neisseria gonorrhoeae*, which is not known to regulate its competence, and is able to acquire DNA during all phases of growth (21). Recently, interest has risen in the consequences of horizontally transferred DNA on the evolution of microbial pathogens (2).

Populations of plant pathogenic bacteria, especially those colonizing crops, are faced with unique environmental pressures that impact the genetic diversity observed in populations. Because host plants tend to be genetically similar, pathogens may undergo periodic selective sweeps where allelic diversity is reduced or eliminated by the emergence of a genotype with increased fitness (18). In practice, such a process mimics the effects of bottlenecks on genetic diversity, as clonal individuals with a shared common ancestor dominate the population. Host-specialized plant pathogens may undergo selective sweeps, eventually embarking on a co-evolutionary arms race with host plants (11). However, a different scenario is plausible for generalist plant pathogens. For these organisms, homologous recombination may lead to the generation and maintenance of allelic diversity in populations (22), which would potentially permit them to explore a wider variety of host plants. While natural competence has been observed in the generalist plant pathogen *Ralstonia solanacearum* (4), it has not been documented in other bacterial plant pathogens. However, there is mounting evidence that recombination of homologous DNA acquired through natural transformation and other

mechanisms could play a much larger evolutionary role than previously thought. Recombination has been shown to increase the rate of pathogen adaptation (3), and emerging diseases have been attributed to the horizontal transfer of virulence factors (19).

Xylella fastidiosa is a plant-pathogenic bacterium that colonizes the xylem vessels of a wide range of host plants and the foregut of its leafhopper vectors (6). Pathogenicity apparently results when *X. fastidiosa* reaches high population density and moves between xylem vessels, inhibiting the flow of xylem sap and leading to symptoms such as leaf-scorching and stunted growth (31). *X. fastidiosa* colonization of plants is strain and host species dependent. In some cases the bacterium multiplies, moves systemically, and induces disease symptoms. In other hosts, *X. fastidiosa* multiplies somewhat but does not move systemically, while the bacterium may move systemically but not cause disease in yet another group of host plants (36). *X. fastidiosa* is the causative agent of Pierce's disease in grapevines in addition to several emerging diseases in other plant hosts, such as citrus variegated chlorosis, and coffee and oleander leaf scorch (23). Recent studies have shown evidence of recombination between different strains of *X. fastidiosa* (1, 40, 46). In addition, MLST studies have indicated that horizontally acquired sequences may play a significant role in introducing genetic diversity to *X. fastidiosa* populations, potentially being more important than point mutations (1, 40). Scally et al. (40) estimated the ratio of the contribution towards diversity of recombination and point mutations (r/m) in *X. fastidiosa* to be 3.23 at the nucleotide level.

Based on increasing evidence that recombination significantly contributes to the genetic diversity of *X. fastidiosa*, we hypothesized that this organism can acquire DNA through natural transformation. This study documents natural competence and the homologous recombination of acquired DNA in *X. fastidiosa* and reports on several factors that affect its occurrence.

MATERIALS AND METHODS

Strains, DNA, media and growth conditions. *X. fastidiosa* subspecies *fastidiosa* strains Temecula (43) and STL (16) were used in this study. The knockout mutant of *pglA* (in strain Fetzer), which encodes a polygalacturonase necessary for systemic colonization of grapevines, was obtained from Roper et al. (38); the knockout mutant of *rpfF*, which encodes the synthase for the cell-cell signaling fatty acid molecule *D*iffusible *S*ignaling *F*actor (DSF), was obtained from Newman et al. (33); the knockout mutant of *rpfC*, which encodes a two-component regulatory protein that senses DSF, was obtained from Chatterjee et al. (7). The above mutants are all kanamycin resistant. Strain NS1-CmR, which contains a chloramphenicol resistance cassette inserted in a non-coding region of the genome, was obtained by transforming Temecula with pAX1-Cm from Matsumoto et al. (30). DNA used for transformation experiments consisted of suicide plasmids pAX1-Cm (30) and pKLN61 (33), or an approximately 2 kb linear segment of pKLN61 containing the kanamycin resistance cassette PCR-amplified using primers rpfF-fwd and rpfF-rev (Table 1). All strains were grown in PWG or PW (liquid medium without solidifying agent) (13), or XFM (25) with the following modifications: 1g/L K_2HPO_4 and 0 g/L KH_2PO_4. Preliminary data suggested that transformation with pKLN61 is more efficient in modified XFM. When appropriate, kanamycin was added to a final concentration of 30 μg/ml and chloramphenicol was added to a final concentration of 10 μg/ml.

General transformation protocol. *X. fastidiosa* cells were harvested from solid PWG medium after approximately 7 days of growth and diluted in 200 μl modified XFM to a final OD_{600} between 0.0025 and 0.05 (approximately 10^6 to $2x10^7$ cfu/ml). After two days of growth at 28°C with constant shaking at 180 rpm, DNA was added to a final concentration of 5μg/ml. Cultures

were then grown for an additional 24 hours and plated on selective media. Antibiotic resistant colonies were counted after approximately 14 days of growth, and recombination events were confirmed through PCR analysis of random samples. Primers rpfF-fwd and rpfF-rev (Table 1) were used to confirm the presence of the kanamycin resistance cassette in the *rpfF* region of the genome, and primers NS1-f and NS1-r (30) were used to confirm recombination between pAX1-Cm and the NS1 region of the genome.

DSF detection assay. Detection of DSF in the *X. fastidiosa* growth medium was conducted using the *Xanthomonas campestris* GFP-based reporter *Xcc* 8523 (pKLN55) (33), which expresses GFP in the presence of DSF. DSF was extracted from 10 PWG plates each grown for one week with wild-type Temecula, the *rpfF* mutant, or recovered antibiotic-resistant cells transformed with either pKLN61 or the linear, PCR-amplified fragment. DSF was extracted essentially as described in Newman et al. (34). Plates were homogenized with 100ml of water-saturated ethyl acetate and incubated for one hour at room temperature. The supernatant was collected and evaporated, and DSF was recovered from the ethyl acetate precipitate into 1 ml of methanol. DSF extract (50 µl) from each sample was spotted on a paper disk on a King's B agar (KB) plate and 100 µl aliquots of the *Xcc* 8523 (pKLN55) streaked perpendicular to the disk. The plates were incubated overnight at 28^{o}C, and fluorescence was viewed on a Zeiss SV11 stereoscope with Kramer epifluorescence/ Optronix Color DEI450.

Cell density recombination experiments. To determine the effect of cell culture age on recombination, *X. fastidiosa* cells were diluted to an OD_{600} of approximately 0.007 in both modified XFM and PW. pKLN61 was added to a final concentration of 5µg/ml after 2, 4, 6 or 8 days of growth. Cultures were grown for an additional 24 hours, and 50 µl aliquots were plated on PWG and PWG with kanamycin. Antibiotic-resistant colonies were counted approximately 14 days after plating. To quantify growth of *X. fastidiosa* in modified XFM, cultures with an initial OD_{600} of 0.005 were grown in triplicate at 28^{o}C with constant shaking at 180 rpm. Samples were taken at day 0, 1, 2, 3, 5, 7 and 9 and total cell count was estimated using quantitative PCR as described below. To determine the effect of initial cell density on recombination efficiency, cultures were prepared as described above, with initial OD_{600}s of 0.0025, 0.005, 0.01, 0.025 and 0.05. pKLN61 was added after 2 days of growth, and cells were plated on PWG with kanamycin after an additional 24 hours. Samples were also taken for quantitative PCR to determine the total number of cells present. Six replicates were used for the recombination experiments.

Recombination between *X. fastidiosa* strains *in vitro*. The *rpfF*, *rpfC*, and *pglA* mutants, which are kanamycin resistant, were each individually grown in triplicate with strain NS1-CmR, which is chloramphenicol resistant, in test tubes with 3ml of modified XFM. The mutant strains and NS1-CmR were each diluted to OD_{600} 0.005 in the same tube, for a total initial OD_{600} 0.01. Cultures (initial OD_{600} 0.01) of *rpfC*, *rpfF*, and *pglA* mutants and NS1-CmR were also grown individually as controls. Cultures were grown for 3 days at 28^{o}C with constant shaking at 180 rpm. Fifty µl aliquots from 0 and 3 days were plated on PWG with kanamycin, PWG with chloramphenicol, and PWG with kanamycin and chloramphenicol. Population sizes at each time point were estimated with quantitative PCR (see below). Colonies that were resistant to both kanamycin and chloramphenicol were PCR screened for the presence of the antibiotic-resistance cassettes in the appropriate loci using primers pglA-fwd and -rev, rpfF -fwd and -rev, rpfC -fwd and -rev (Table 1), and NS1-f and NS1-r (30).

***X. fastidiosa* cells and extracellular DNA quantification.** Quantification of *X. fastidiosa* cells and extracellular DNA was performed using quantitative PCR. For cell

quantification, aliquots of culture were heated at 99°C for 15 minutes, and 1 µl of the suspension was used as the template for quantitative PCR using Applied Biosystem's SybrGreen Master Mix with 0.5 µM each of primers HL5 and HL6 (17). Reactions (absolute quantification) were run on Applied Biosystems Fast7500 in standard mode with the following cycle: 55°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Fluorescence was quantified at 60°C. Standards were created using cells harvested from PWG and diluted in modified XFM. Cell counts for standards were determined based on OD_{600}. To quantify extracellular DNA, the culture was passed through a 0.2 µm filter to remove cells, and 1 µl of filtrate was used as the template. Standards were created with genomic Temecula DNA quantified on a Thermo Scientific Nanodrop 1000 Spectrophotometer.

Transformation efficiency of methylated plasmids. Previous work has shown that *X. fastidiosa* has a functional restriction modification system, and properly methylated plasmids recombine at higher efficiencies when introduced through electroporation (29). We transformed *Escherichia coli* strain EAM1(29), which expresses the *X. fastidiosa* methylase PD1607, with pKLN61 using the Z-Competent *E. coli* Transformation Buffer Set from Zymo Research (Irvine, CA). Cells were then grown overnight in LB with 1mM IPTG to induce expression of the methylase. Transformation experiments were carried out as described with a starting OD_{600} 0.01.

RESULTS

***X. fastidiosa* is naturally competent.** *X. fastidiosa* strain Temecula was able to naturally transform and homologously recombine exogenous DNA into its genome when grown in modified XFM liquid medium without selection. After adding pKLN61, a plasmid that cannot replicate in *X. fastidiosa* containing portions of *rpfF* flanking a kanamycin resistance cassette, or a linear PCR-amplified copy of the gene and cassette, we recovered antibiotic resistant colonies. PCR analysis confirmed that the antibiotic resistant colonies were a result of a double recombination event between the genome and plasmid/linear DNA (Fig 1A). Amplification of the *rpfF* locus of the transformed colonies and the *rpfF* mutant (control) resulted in a single amplicon approximately 800 bp longer than the wild type copy. Complete inactivation of the *rpfF* gene was also confirmed using *X. campestris* strain *Xcc* 8523 (pKLN55) that expresses GFP in the presence of DSF (Fig 1B,C,D).

The transformation protocol was also performed using strain STL, and different plasmids in Temecula to show that the phenomenon of natural transformation and recombination is not strain or sequence specific. STL was successfully transformed with pKLN61 and the linear PCR amplified fragment. Temecula was also successfully transformed with pAX1-Cm (30), which inserts a chloramphenicol resistance marker in a non-coding region of the genome, and several other novel plasmids. PCR analysis confirmed clean double recombination events in all cases (data not shown).

Cell growth affects recombination efficiency. The maximum number of recombinants, as determined by acquired antibiotic resistance, was obtained when DNA was added after two days of growth on modified XFM. Transformation and recombination rates decreased with time after inoculation. Cells were grown for 2, 4, 6, or 8 days before pKLN61 was added. No antibiotic resistant colonies were recovered when DNA was added after 8 days of growth (Fig 2A). Cells were also grown in PW medium with pKLN61, but no recombinants were recovered at any time point. In addition, no antibiotic resistant colonies were recovered for the control group when no DNA was added. However, all treatments grew on PWG without antibiotics, confirming that cell cultures were still viable. A growth curve for cells in XFM shows that there

is an initial two day lag phase under experimental conditions, and the cells enter stationary phase within seven days after inoculation (Fig 2B). Cells appear most competent as they enter exponential growth, as the number of recombinants recovered drops dramatically when DNA is added after 4 and 6 days, despite the fact that more cells are present.

To determine if cell density was solely responsible for the decrease in antibiotic resistant colonies recovered over time, the recombination efficiency (number of recombinants recovered divided by the final cell count) for cell cultures with different initial optical densities was measured. Cell cultures with initial OD_{600}s varying from 0.0025 and 0.05 were given pKLN61 as described. No recombinants were recovered from cultures with an initial optical density of 0.0025 (Fig 2C). For cultures with higher starting optical densities, however, there was an increase in the total number of recombinants recovered per 50 µl. An analysis of variance and Tukey statistical test run on the log-transformed recombination efficiencies indicates that the efficiency for OD_{600} 0.0025 was significantly lower than all other optical densities ($p= 2.2 \times 10^{-16}$), but the difference in recombination efficiencies between the other treatments was not significant (Fig. 2D). Overall recombination efficiencies were on the order of 4 to 10 recombinants for every 10^6 cells present.

By comparing the starting and final cell counts for each of the cultures, the data show that the number of generations differed depending on the starting optical density. For cultures with a starting OD_{600} of 0.0025, there was an average of 1.3 generations over the course of three days (Fig. 2D). The average number of generations peaked at 3.4 for cultures with an initial optical density of 0.01. Growth appeared to be slightly inhibited when cells were started at the highest OD_{600} of 0.05, as the total number of generations over three days decreased to approximately 2.4. Cultures started with OD_{600} 0.0025 had significantly fewer generations over the course of three days than all other treatments. Cultures started with an OD_{600} 0.05 also underwent significantly fewer generations than cultures with an initial OD_{600} 0.01. There was no significant difference in any of the other treatments. The correlation coefficient between the log transformed recombination efficiency and number of generations was $r = 0.826$ with $p<0.001$ (28 degrees of freedom).

Recombination between *X. fastidiosa* strains *in vitro*. Recombination occurred between strains of *X. fastidiosa* when different strains were co-cultured. *X. fastidiosa* knockout mutants of *rpfF, rpfC,* and *pglA* (all are kanamycin resistant) were individually inoculated with strain NS1-CmR (chloramphenicol resistant) in XFM liquid medium and grown for 3 days at 28°C. Recombination between the mutant and NS1-CmR was quantified by counting the number of doubly-antibiotic resistant colonies (Fig 3). PCR analysis confirmed site-specific recombination by the presence of a single, antibiotic-resistant allele at each of the appropriate loci. Recombination was observed in approximately 4 out of every 10^7 cells for the *pglA* mutant and NS1-CmR, and 9 out of every 10^7 cells for the *rpfC* mutant and NS1-CmR. No recombination was detected between the *rpfF* mutant and strain NS1-CmR. An analysis of variance test indicated that the three treatments were significantly different ($p= 0.018$), but a multiple comparisons of means (Tukey) indicated the difference in recombination efficiencies between mutants *rpfC* and *pglA* with NS1-CmR was not significant ($p= 0.13$).

Extracellular DNA present in the media at the start of the experiment and after three days of growth was also quantified. Aliquots of each culture at 0 and 3 days were filtered through a 0.2 µm filter to remove cells, and total extracellular DNA was estimated using quantitative PCR. The amount of extracellular DNA increased after 3 days for all three treatments—approximately 15, 36, and 37 times for the *rpfC, rpfF,* and *pglA* mutants grown with NS1-CmR respectively.

There was no significant difference in the amount of extracellular DNA present in the different treatments after three days of growth (p= 0.099), with the final concentration 6 to 22 picograms per microliter. In addition, there was no significant difference in the recombination efficiency when measured in terms of extracellular DNA concentration (p= 0.170; Fig 3).

Transformation and recombination of methylated DNA is more efficient. As previous work has indicated that *X. fastidiosa* has a functional restriction modification system (29), the recombination efficiencies of cells transformed with pKLN61 and *X. fastidiosa*-specific methylated pKLN61 were compared. Significantly more recombinants were recovered from cells transformed with the methylated plasmid than the unmethylated plasmid (average of 28.3 ± 5.15 and 6.3 ± 1.84 per 50 μl of culture, p= 0.003).

DISCUSSION

X. fastidiosa appears to exchange DNA at relatively high rates when different strains are grown together *in vitro*, and actual recombination rates between strains were likely higher than observed. High recombination rates between co-cultured strains has also been observed in other bacteria; recombination in mixed cultures of *Streptococcus mutans* and *Streptococcus gordonii* was observed in approximately one out of every 10^6 viable cells after four hours of incubation (26). As recombination between the *X. fastidiosa* strains occurred before the cells were subjected to selective pressure on the markers, and assuming recombination occurs randomly between homologous regions of the genome, one can postulate that only somewhere on the order of 0.1% of total events (based on *X. fastidiosa*'s approximately 2.5 Mb genome and the 1 kb antibiotic marker) were detected. Previous work with *Helicobacter pylori* showed that recombination of naturally transformed DNA in approximately one out of every 10^7 cells per passage (about 5.5 generations) was sufficient to confer a fitness advantage over non-competent cells (3). Data indicate that natural transformation and recombination rates in *X. fastidiosa* are at least that high, suggesting that recombination can alter *X. fastidiosa*'s genome and increase fitness and adaptation on a larger and more rapid scale than mutations alone.

Growth stage has been shown to be one of many factors regulating competence in bacteria, although there does not seem to be a single trend. In *Streptococcus pneumoniae,* for example, competence is inhibited during stationary phase, while in *Bacillus subtilis*, competence does not develop until its onset (10). Based on growth curve data, *X. fastidiosa* appears to be most competent as it is entering exponential growth, similar to *R. solanacearum*, where the onset of competence occurs near the beginning of exponential growth and quickly declines during log phase; cells are essentially not competent once they reach stationary phase (4). This is also confirmed by the significant correlation found between the log transformed transformation efficiencies and generations. The correlation suggests that the increase in the number of recombinants recovered from cultures that had undergone more generations was at least partly due to previously transformed cells multiplying (as opposed to new cells being transformed). Testing the effect of different starting optical densities on the transformation and recombination rates revealed that cell density does not seem to directly affect transformation rates on the time scale tested, but that cells must be dividing in order for recombination to occur. It is possible that no recombination and little growth for cultures with starting OD_{600} 0.0025 was observed because there was an extended lag phase due to the low cell density. However, cell-cell signaling may still be important, as recombination was not detectable between the *rpfF* mutant and NS1-CmR when the two strains were co-cultured in modified XFM. Quorum-sensing has

been shown to control competence in other bacteria (44), so it is possible that it at least partially regulates this process in *X. fastidiosa*.

In addition, we found that *X. fastidiosa* is competent when grown in a nutrient-limited, defined growth medium (modified XFM), but cannot transform and recombine exogenous DNA when grown in an undefined, rich medium (PW). Competence in other bacteria is often controlled by factors such as growth stage and nutritional signals (8). This seems to be the case for *X. fastidiosa* as well. The fact that *X. fastidiosa* appears to be most competent when it is undergoing rapid growth in low-nutrient conditions lends support to the hypothesis that natural transformation may be the result of cells importing nucleotides for nutritional purposes (37).

Data also indicate that extracellular DNA concentration increases in cultures of *X. fastidiosa*. There are several possible reasons and mechanisms for this increase. It has been suggested that extracellular DNA plays a role in *X. fastidiosa* biofilm formation (9). Studies of *S. pneumoniae* have shown that when cells become competent, they can induce the lysis of non-competent cells in the population to provide a source of nucleotides (10). Other naturally competent bacteria, such as *N. gonorrhoeae*, can donate DNA for transformation through type IV secretion systems (21). However, it is not known if the extracellular DNA in the case of *X. fastidiosa* is the result of dead cells with compromised membranes, competent cells actively inducing the lysis of other cells, or live cells secreting DNA. If one assumes that all actively growing *X. fastidiosa* cells increase the concentration of extracellular DNA in their immediate environment, recombination between different strains could happen readily in natural environments, such as in plants or insect vectors, whenever two different strains come in contact with each other. There is evidence that mixed infections can exist in *X. fastidiosa*'s sharpshooter vectors (12), providing a possible means and location for recombination to occur in natural environments. MLST studies showing that recombination occurs between different strains of *X. fastidiosa* support this hypothesis (1, 40, 46). The fact that extracellular DNA concentrations probably increase during growth could affect our total cell quantification, as it was assumed that the DNA detected by quantitative PCR was from intact cells. However, if the number of cells present has been overestimated, then recombination rates would be even higher than reported.

Recombination rates were about 5 times higher when cells were transformed with *X. fastidiosa*-specific methylated plasmids than when cells were transformed with unmethylated DNA. Similar increases in recombination rates were found when methylated and unmethylated plasmids were introduced into *X. fastidiosa* by electroporation (29). Restriction modification plays a large role in DNA processing in many naturally competent bacteria; *N. gonorrhoeae* encodes approximately 16 methyltransferases in its genome, and with the corresponding endonucleases, acts as a restriction barrier to transformation of foreign plasmids. There is no similar restriction on genomic DNA, and transformation efficiencies can be 1000-fold higher than for foreign DNA (21). There is also evidence that the presence of different restriction modification systems coincides with the structure of phylogenetic clades in *Neisseria meningitidis* and could account for the differential barrier to DNA exchange observed within the species (5). Restriction modification systems could help explain how such high recombination rates were observed when *X. fastidiosa* strains were grown in co-culture, despite the fact that extracellular DNA concentrations were low. Non-methylated or improperly methylated DNA, such as what was used in initial experiments, may be subject to *X. fastidiosa*'s restriction modification system and degraded before it can be recombined into the genome. Transformation experiments had approximately 1,000-fold more DNA added than was found in the co-culture media. In addition, if the extracellular DNA released in co-culture is simply genomic DNA, then

less than one-thousandth of the potentially transforming DNA contained the selectable marker. Thus, it appears that *X. fastidiosa* cells are able to take up and recombine DNA originating from *X. fastidiosa* at much higher rates than DNA from other sources. The active restriction modification system and the fact that *X. fastidiosa* has historically been grown in nutrient-rich media could help explain why this bacterium has been difficult to transform using conventional methods.

The experiments performed here provide evidence that DNA can enter *X. fastidiosa* cells through natural transformation and be homologously recombined into the genome. None of the DNA sources used could independently replicate in *X. fastidiosa*, so the antibiotic markers could not have been conjugally transferred between cells on a plasmid. Recent work has suggested that certain strains of *X. fastidiosa* harbor plasmids that encode conjugal transfer proteins and could potentially be horizontally transferred through conjugation (41), but in our case, the mechanism of horizontal gene transfer is transformation. A search of the *X. fastidiosa* genome found many genes and putative genes that would allow for the uptake and recombination of exogenous DNA (Table 2). Based on the mechanism of transformation in *N. gonorrhoeae* (21), a naturally competent Gram-negative bacterium, we propose that transforming DNA crosses the outer membrane of *X. fastidiosa* through a PilQ or PilQ-like channel that is part of a type IV or similar pilus. A type IV pilus or a pilus-like structure is required for DNA uptake in nearly all naturally transformable bacteria, as are a series of Com proteins (8). Recent work has shown that *X. fastidiosa* has functional type IV pili (14, 27), and a search of the *X. fastidiosa* genome produced several annotated *com* genes (24). The different *com* genes facilitate DNA binding and transportation into the cytoplasm. In *N. gonorrhoeae*, ComE binds the transforming DNA in the periplasm and is necessary for efficient uptake, ComL is directly or indirectly involved in puncturing the peptidoglycan layer, and ComA is an inner membrane protein that helps transport DNA into the cytoplasm (21). Once inside the cytoplasm, *X. fastidiosa* has the machinery necessary for RecA-mediated recombination.

Initially, it was thought that most plant pathogenic bacteria, such as *Pseudomonas syringae*, were primarily clonal (39). However, recent studies have provided evidence indicating that limited and biased sampling within populations may have underestimated the rate of homologous recombination in these organisms (20, 45). In addition, several highly-specialized pathogens recombine at much lower frequencies than their non-specialized relatives, and it has been hypothesized that the emergence of new pathogens could be characterized by high recombination rates during adaptation to new environments or hosts, followed by a drop in recombination frequency once the pathogen has settled into its new niche (15). Recent studies have shown that *X. fastidiosa*'s genome is subject to recombination, and that this process could play a large role in generating genetic diversity and affect the organism's evolution (1, 40, 46). It has also been hypothesized that Pierce's disease-causing strains of *X. fastidiosa* in the United States, which have little allelic diversity, diverged from an isolate introduced from Costa Rica (35). If true, these recently diverged strains could rapidly develop novel genotypes as they recombine with endemic strains, though this has not yet been shown to happen. Here, evidence has been provided that *X. fastidiosa* is naturally competent, and that transformation is a likely mechanism for DNA to be horizontally transferred between organisms. Further studies of *X. fastidiosa*'s natural competency could provide insight into the effects of recombination on pathogen diversity and the emergence of new diseases.

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Table 1. Primers used in this study.

Primer function	Primer name	Sequence	Source
Flanking *rpfF*	rpfF-fwd	5’ TGGAGTGGTGTGCTCTTGTCCA	This study
	rpfF-rev	5’ ACGCGATACGGAAGTACCACCA	
Flanking *rpfC*	rpfC-fwd	5’ AGCTTTTGGTGTTGCTGTCCG	This study
	rpfC-rev	5’ GCTTTCATCGTAAAACCCCACTG	
Flanking *pglA*	pglA-fwd	5’ AATCGCTCAGCTTCAGTCCG	This study
	pglA-rev	5’ GCCCTCCAGTGAAGGAATTTCT	
Flanking NS1	NS1-f	5’ GTCAGCAGTTGCGTCAGATG	(30)
	NS1-r	5’ AAAGCTGCCGACGCCAAATC	
X. fastidiosa quantification	HL5	5’ AAGGCAATAAACGCGCACTA	(17)
	HL6	5’ GGTTTTGCTGACTGGCAACA	

Table 2. Annotated *com* genes present in the *X. fastidiosa* strain Temecula genome.

Locus	Name	Putative Function
0031	*comJ*	transformation competence related protein
0042	*comF*	competence protein F
0358	*comA*	DNA uptake protein
0464	*comM*	competence-related protein
1558	*comE*	DNA transport competence protein
1756	*comL*	competence lipoprotein

Fig. 1.

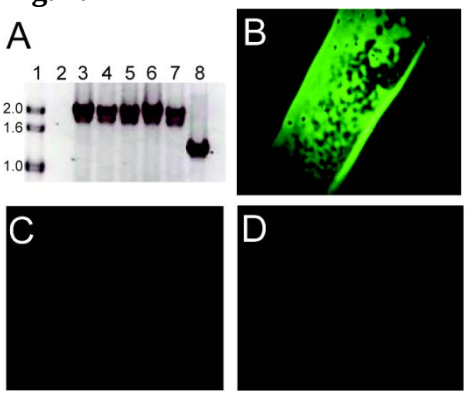

Fig. 1. Confirmation of natural competence and recombination in *X. fastidiosa*. (A) Size standards (lane 1) from top to bottom: 2, 1.6 and 1 kb. PCR of a negative control (lane 2) and genomic DNA extracted from colonies transformed with pKLN61 (lanes 3 and 4), linearized *rpfF:kanR* (5 and 6), the *rpfF* mutant (7), and wild-type Temecula (8) was performed using primers rpfF-fwd and rpfF-rev. The wild type copy of *rpfF* is approximately 1.2kb, and the mutant copy is approximately 2kb. Fluorescence microscopy images of bioassay detection for presence of DSF in extracts taken from wild type cells (B), colony transformed with pKLN61 (C), and *rpfF* mutant (D). GFP expression by *Xcc* 8523 indicates the presence of DSF, which is not produced by the strain transformed with pKLN61 or the *rpfF* mutant.

Fig. 2.

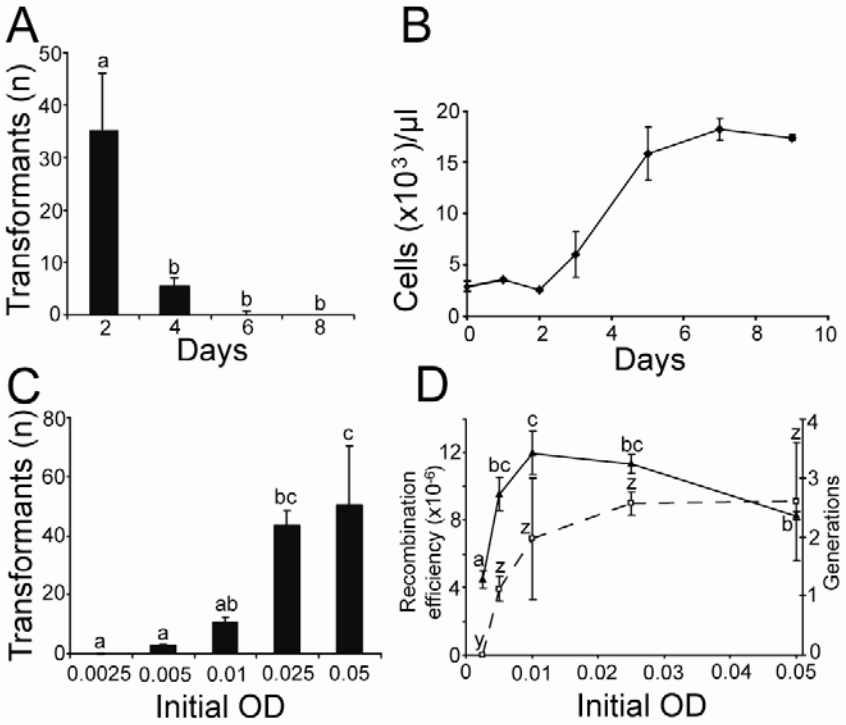

Fig. 2. Growth effects on recombination efficiency. (A) The number of recombinants decreased over time as pKLN61 was added after 2, 4, 6 or 8 days of growth. Cultures were plated on selective media after a 24 hour incubation period with transforming DNA. Recombination events were quantified based on the number of antibiotic-resistant colonies present after approximately 14 days of growth. (B) Growth curve of cells in XFM. (C) Total number of recombinants recovered from cultures with different starting optical densities. Cultures were grown for 2 days before the addition of pKLN61 and grown for an additional 24 hours before plating on selective media. (D) Recombination efficiency (squares, dashed line) and number of generations over 3 days (triangles, solid line) for cultures with different starting ODs. The correlation coefficient between the log-transformed recombination efficiencies and number of generations is r= 0.826 ($p<0.001$). Different symbols/letters indicate statistically different groups ($p<0.05$).

Fig. 3.

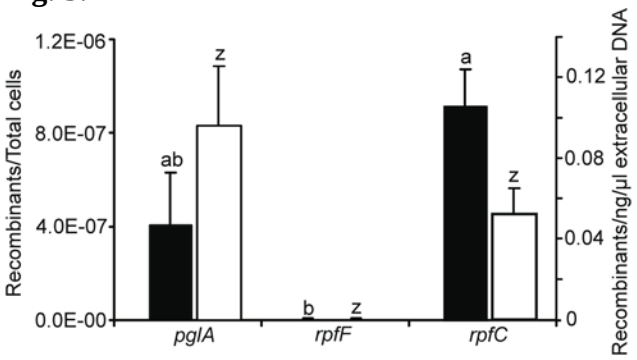

Fig. 3. Recombination efficiencies between different strains grown in co-culture. Mutant strains *pglA*, *rpfF*, and *rpfC* were grown individually in co-culture with NS1-CmR in XFM liquid medium for three days. Recombination efficiencies between strains are shown based on the number of colonies resistant to both kanamycin and chloramphenicol per total number of cells present (solid bars) and nanograms of extracellular DNA in the medium (empty bars). Recombination events were also confirmed by PCR analysis. Letters indicate statistically significant differences between groups ($p<0.05$).

Chapter 3: Effects of DNA size on transformation and recombination efficiencies in *Xylella fastidiosa*

ABSTRACT

Horizontally transferred DNA acquired through transformation and recombination has the potential to contribute to the diversity and evolution of naturally competent bacteria. However, many different factors affect the efficiency with which DNA can be transformed and recombined. In this study, we determine how the size of both homologous and non-homologous regions affects transformation and recombination efficiencies in *Xylella fastidiosa*, a naturally competent generalist pathogen responsible for many emerging plant diseases. Our experimental data indicate that 96 bp of flanking homology is sufficient to initiate recombination, with recombination efficiencies increasing exponentially with size of homologous flanking region up to 1kb. Recombination efficiencies also decreased with size of the non-homologous insert, with no recombination detected when 6kb of non-homologous DNA was flanked on either side by 1kb of homologous sequences. Upon analyzing sequenced genomes from subspecies *fastidiosa* for evidence of allele conversion, we estimated the mean size of recombination events to be 1906 bp, with each event modifying on average 1.79% of the nucleotides in the recombined region. There is increasing evidence that horizontally acquired genes significantly affect the genetic diversity of *X. fastidiosa*, and DNA acquired through natural transformation could be a prominent mode of this horizontal transfer.

INTRODUCTION

Homologous recombination is an important mechanism by which horizontally-transferred DNA is incorporated into the genome of the recipient organism. In bacteria, DNA can be horizontally acquired through three main mechanisms: transformation, conjugation, and transduction. Transformation is a likely route for DNA acquisition for naturally competent taxa, which are found in a wide range of phyla (1). Once inside the cell, DNA can be incorporated into the genome or degraded; it has been hypothesized that natural competence originally evolved as a nutrient uptake system (2). However, there is potential for horizontally acquired DNA to alter the recipient's fitness and phenotype if the DNA is recombined into the genome. Horizontal gene transfer through natural transformation can increase the rate of adaptation of pathogens to new environments (3). Natural transformation increased the pathogenicity of a *Ralstonia solanacearum* isolate when it recombined with DNA from a highly virulent strain (4). Furthermore, it has been shown that adding a single gene in *Vibrio fischeri* is sufficient to alter its host range (5). Thus, horizontal transfer of even small segments of DNA can have significant evolutionary effects.

Several factors can limit horizontal gene transfer through natural transformation and recombination. Gene transfer between organisms can be hampered by geographic separation. The lack of DNA uptake sequences (DUS), short DNA sequences recognized and preferentially bound by competent cells, in donor DNA can prevent the recipient from efficiently binding and transporting the DNA across the membrane (6). Restriction enzymes can degrade un- or improperly methylated DNA in the cytoplasm before it is recombined into the genome (7, 8). In addition, sequence similarity and size of transforming DNA can strongly regulate recombination efficiency. In several organisms across domains, recombination efficiency decreases exponentially with sequence divergence (9–13). However, in cases where sexual isolation is caused by the difficulty of strand invasion of highly divergent sequences, the presence of

flanking regions of identity can remove most recombinatorial barriers (10). The minimal efficient processing segment (MEPS), or the shortest length of sequence homology necessary for efficient recombination, can vary greatly depending on the organism, the recombination pathway used, and other factors. In *Escherichia coli*, efficient recombination has been observed with as few as 23 base pairs of sequence homology (13). The MEPS requirements for eukaryotes has been studied extensively, revealing great variation in this process: recombination efficiency in *Trypanosoma brucei* requires 50 bp of homology for efficient recombination, while essentially no recombination was observed in *Saccharomyces cerevisiae* with fewer than 248 bp of flanking homology (14, 15). The total size of transforming DNA can also affect recombination efficiency; *R. solanacearum* can naturally transform and recombine 90kb of DNA, but efficiencies were three orders of magnitude lower than when the transforming DNA was 1kb in length (16).

Xylella fastidiosa is a plant-pathogenic bacterium that colonizes the xylem vessels of its host plants and inhibits the flow of xylem sap, resulting in symptoms such as leaf scorching and stunted growth (17). It is the causative agent of several economically important diseases, such as Pierce's disease of grapevine, citrus variegated chlorosis, and coffee leaf scorch (18). Recently, this bacterium was shown to be naturally competent and able to homologously recombine acquired DNA into its genome in laboratory environments (19). Multi-locus sequence typing (MLST) studies have also shown evidence of recombination between different strains of *X. fastidiosa*, with horizontally acquired sequences potentially playing a greater role in generating diversity than point mutations (20–22). The different strains and subspecies of *X. fastidiosa* share a highly conserved core gene pool containing genes likely responsible for adaptation to life in the plant xylem, but also have a highly diverse flexible gene pool, potentially responsible for its wide host range (23). However, it is also possible that different alleles, as opposed to unique genes, are responsible for the varied phenotypes within *X. fastidiosa*: altering gene expression in one strain of *X. fastidiosa* increased its virulence to a different host plant (24). Recently emerged pathogenic strains of *X. fastidiosa* may have resulted from the horizontal transfer of elements in the flexible gene pool present in endemic populations to strains introduced from new geographic regions (23).

There is increasing evidence that horizontally-acquired DNA affects the evolution of *X. fastidiosa* with significant ecological consequences and that natural transformation is a likely route for this to occur. This study examines how the characteristics of transforming DNA affect *X. fastidiosa*'s ability to naturally transform and recombine it into its genome. We examined this process in two different contexts: the integration of novel DNA experimentally, and its occurrence in natural populations, as evident from genome sequence comparisons. The first process provides insight into how the flanking homologous region affects recombination efficiency and how much novel DNA can be inserted by recombination, neither of which has been studied extensively in bacteria, while the second shows the extent of the effects of recombination in a population of *X. fastidiosa.*

METHODS

Strain, media and growth conditions. The *X. fastidiosa* subspecies *fastidiosa* strain Temecula (25) was used in this study. Cells were grown in either periwinkle wilt Gelrite (PWG) medium (26) or in modified *X. fastidiosa* medium (XFM) (19). Where appropriate, kanamycin was added to a final concentration of 30µg/ml. We used *Escherichia coli* strain EAM1 (8), which expresses an *X. fastidiosa* methylase, to propagate plasmids. Previous work has shown that

transformation and recombination efficiencies are higher for methylated plasmids than un-methylated plasmids (8, 19).

Plasmid construction. Plasmids for testing the effect of both flanking homology length and insert length were created using the pGEM-5zf (+) vector backbone (Promega, Madison, WI), which cannot independently replicate in *X. fastidiosa*. Flanking homology length plasmids were created by amplifying genomic DNA from an *rpfF* mutant (27) using primers annealing approximately 26, 35, 50, 96, 200, 508, 760, 1kb, 2kb or 4kb upstream and downstream of the kanamycin marker with a *Sac*I restriction site engineered into the 5' end of each primer (Table 1). PCR constructs and the vector backbone were digested with SacI and ligated to create p26, p35, p50, etc. (Fig. 1). Plasmids were transformed into *E. coli* strain EAM1 for propagation as previously described (19).

To construct plasmids with different lengths of non-homologous DNA, we first used primers F1 fwd *Sph*I and F1 rev *Nco*I (Table 1) to amplify the kanamycin cassette and approximately 1 kb of DNA upstream of the KanR insertion site within *rpfF* mutant. This was digested with *Sph*I and *Nco*I and ligated into pGEM-5zf(+) to create pS1. Approximately 1kb of flanking DNA immediately downstream of the kanamycin marker in the *rpfF* mutant was amplified using F2 fwd *Sal*I and p1k-r (containing a *Sac*I site at the 5' end). We then digested this fragment and pS1 with *Sal*I and *Sac*I and ligated the two together to create pS2. Non-homologous insert DNA ranging in size from 1 to 5kb with an *Nco*I site at one end and a *Not*I site at the other was amplified from cDNA from a single-stranded positive-sense RNA plant virus with no homology to any region of the *X. fastidiosa* genome (Genbank accession number JQ655296.1). All fragments were amplified from the region of approximately base pair 3100 to 8100 using the primers listed in Table 1. PCR products and pS2 were digested with *Nco*I and *Not*I and ligated together to form pS2-1k, pS2-2k, pS2-3k and pS2-5k (Fig. 2B). Plasmids were transformed into *E. coli* strain EAM1 for propagation.

Transformation protocol. *X. fastidiosa* cells were harvested from PWG plates after approximately 5-7 days of growth and resuspended in 200µl of XFM to a final OD_{600} of approximately 0.01. After 2 days of growth at 28^{o}C with constant shaking, we added the appropriate plasmids to a final concentration of 5µg/ml. After an additional 24 hours of growth, cultures were plated on PWG with kanamycin and an aliquot was frozen for quantification. We counted antibiotic-resistant colonies after approximately 14 days of growth. We confirmed the insertion of the antibiotic marker and additional non-homologous DNA (if appropriate) at the correct locus occurred by double recombination events through PCR analysis of a random sample of antibiotic resistant colonies using primers rpfF-fwd and rpfF-rev (19), which anneal approximately 730 bp upstream and 350 bp downstream of the putative kanamycin and non-homologous DNA (if appropriate) insertion site. These primers produce an amplicon of about 1200bp in cells with the wild type locus and 2kb in cells with the kanamycin cassette inserted in the proper location. Additional non-homologous DNA from the plasmids was also amplified by these primers, with recombination between pS2-1k producing a fragment of approximately 3 kb, pS2-2k producing a fragment of 4kb, etc. Cells were quantified using qPCR as previously described (19). Recombination efficiencies were calculated based on the number of antibiotic-resistant colonies divided by the total number of cells present. Fifteen replicates for each plasmid were used to measure the effect of insert length on recombination efficiencies; 6 to 15 replicates were used to test each plasmid with different homologous flanking regions. The estimated limit of detection was calculated as previously described (28).

Genome comparisons. Homologous recombination events were inferred with the program ClonalFrame (29), which models bacterial evolution as the diversification of a clonal population with polymorphisms arising due to a combination of mutations, which affect single nucleotides, and allele conversions, which affect contiguous regions of the chromosome and modify a small portion of the nucleotides in that region. The analysis included four publicly available chromosome sequences from subspecies *fastidiosa*: Temecula1 (GenBank: AE009442.1), M23 (CP001011.1), GB514 (CP002165.1) and EB92.1 (AFDJ00000000.1). Chromosomes were aligned using progressiveMauve (30), with default settings. Small aligned blocks (<5 kb) were removed with the program stripSubsetLCBs; small alignment blocks are less likely to represent segments of the ancestral chromosome, as reflected in the radically higher levels of polymorphism (data not shown). Conserved coding regions between subspecies *fastidiosa*, *multiplex*, and *pauca*, were identified as reciprocal best BLAST hits between Temecula1, M12 (RefSeq: NC_010513.1, 26-Jan-2012), and 9a5c (NC_002488.3) using the software DNAMaster.

RESULTS

Recombination efficiency depends on homologous sequence length. We observed maximum recombination efficiency of a naturally transformed plasmid into the *X. fastidiosa* genome when the approximately 1kb kanamycin marker was flanked on both sides by 1kb of homologous *X. fastidiosa* DNA. Maximum recombination efficiency (recombinants recovered per total cells present) was 5.62×10^{-5} (Fig. 1A). Overall recombination efficiencies decreased for plasmids with 2kb and 4kb flanking regions, but when efficiencies were normalized for the different plasmid sizes (thus accounting for the total number of plasmids added), there was no significant difference between recombination efficiencies for plasmids with 1kb, 2kb and 4kb of homologous flanking regions ($p=0.025$). An analysis of variance and Tukey ad hoc test indicated the recombination efficiencies for plasmids with 1kb and 4kb of homology were significantly higher than that of plasmids with 96, 200, 508 and 760 bp of flanking homology ($p < 1\times10^{-4}$). When taking into account the size difference of the plasmids, recombination efficiency was reduced by an order of magnitude when the flanking region was decreased from 1000 bp to 760 bp; a further decrease by an order of magnitude occurred between 760bp and 508bp. Recombination efficiency increased exponentially with the length of the flanking region in the range of 508 to 1000 bp ($r= 0.83$, $p<1x10^{-5}$).

In separate trials, we tested the recombination efficiencies of plasmids with flanking regions ranging from 26bp to 1000bp (Fig. 1B). A single instance of recombination mediated by 96 bp of flanking region was observed, but no other instance of recombination of plasmids with less than 508 bp of homology flanking the kanamycin cassette was detected using the methods described here. The estimated limit of detection for recombination rates was approximately $6x10^{-8}$. Random samples of antibiotic resistant colonies from each treatment were PCR analyzed to confirm additive integration at the correct locus occurred by a double recombination event. All amplicon lengths were as expected.

Recombination efficiency decreases exponentially with size of inserted non-homologous DNA. Recombination of pS2, which contained a kanamycin marker flanked on either side by 1kb of homologous DNA, occurred in approximately one out of every 2.24×10^{-6} cells (Fig. 2). Increasing the size of the non-homologous region by 1kb (using plasmid pS2-1k) decreased the recombination efficiency by almost an order of magnitude (4.65×10^{-7}). Recombination efficiencies for pS2-2k and pS2-3k, having an additional 2kb or 3kb of non-

homologous DNA in addition to the 1kb kanamycin marker, were 1.42×10^{-7} and 2.31×10^{-7} respectively. We did not detect recombination between pS2-5k, having a total non-homologous insert size of approximately 6kb, and the *X. fastidiosa* genome. The estimated limit of detection was approximately $9x10^{-8}$.

Recombination occurs in natural populations. The software package ClonalFrame (29) was used to analyze four published genomes within *X. fastidiosa* subspecies *fastidiosa* to detect recombination events resulting in allele conversion. The estimates of ClonalFrame's parameters indicated the ratio of recombination events to mutation events (ρ/θ) was 0.48, while the ratio of nucleotides changed by recombination versus nucleotides changed through mutation (r/m) was 15. This suggests that allele conversion was a major contributor to clonal diversification, occurring half as often as point mutations, but contributing much more to the accumulation of polymorphisms in the core genome of this population. The average size of each allele conversion event was 1906 bp, with 1.79% of the nucleotide sequence changed (95% credibility regions of 1464 to 2392 and 1.71% to 1.87%, respectively). For comparing the diversity of possible donor DNA sequences, we found the nucleotide identity between the shared coding regions of subspecies *fastidiosa* and *multiplex* was 98.4%, and 97% between *fastidiosa* and *pauca*.

DISCUSSION

Horizontal gene transfer plays a large role in generating genetic diversity in a wide range of bacterial species, and natural transformation can be an important way for organisms to acquire novel DNA sequences. The average import size of DNA acquired through transformation for a variety of naturally competent bacteria is in the range of 1-10kb (31–33), although natural transformation and recombination of much larger segments of DNA has been demonstrated (4, 16). Recombination of shorter fragments may be inefficient due to degradation during uptake and processing, or such events could be undetectable. Our experimental data indicated that natural transformation in a population of *X. fastidiosa* is consistent with these size parameters, as was the average size of recombination events as determined by the ClonalFrame analysis. These data support our hypothesis that transformation is an important driver of horizontal gene transfer in *X. fastidiosa*.

To determine size requirements of recombination events in natural isolates, we performed a ClonalFrame analysis on four genomes within *X. fastidiosa* subspecies *fastidiosa*. The small sample size resulted from the lack of full genome sequences available within the subspecies. We could not include genomes from outside subspecies *fastidiosa*, as there is evidence of non-uniform substitution rates between different subspecies (34), which violates one of ClonalFrame's assumptions (29). In addition, including genomes from the entire species led to long branches between subspecies, decreasing the robustness of the results (data not shown). Lastly, large insertion/deletion events in single genomes would not be detected in this analysis as these regions would not align properly (29). The average nucleotide replacement rate for each recombination event was 1.79%, suggesting that *X. fastidiosa* is routinely able to recombine with genomes with 98.2% similarity. Based on the sequence alignments of the shared coding regions of various *X. fastidiosa* genomes, we found that subspecies *fastidiosa* and subspecies *multiplex* share 98.4% sequence identity of their aligned regions, supporting conclusions from previous MLST studies (20–22) that recombination between different subspecies occurs at relatively high frequencies. Since ClonalFrame models recombined fragments as originating from a single population with the estimated level of divergence, it will have largely ignored recombination

events originating from within subspecies *fastidiosa*, which would have altered very few nucleotides. As the biology of the different subspecies varies in terms of pathogenicity and host range (35), recombination could potentially result in the emergence of new strains with markedly different phenotypes.

A log-linear relationship has been established between sequence divergence and recombination efficiencies for a number of recombining bacteria, although the slope of the curve varies between species (9, 11, 36). Decreasing sequence similarity to approximately 90% reduces recombination efficiency by one order of magnitude for *Streptococcus pneumonia* (11), and three orders of magnitude for *Bacillus subtilis* (36). Although we did not determine the actual relationship between sequence similarity and recombination efficiency, the average replacement rate for recombination events in *X. fastidiosa*, which indicates on average 1.79% of the nucleotides in the donor DNA differs from the sequence of the recipient's DNA, is consistent with previously reported data.

Our experimental work determined the parameters needed for efficient additive integration, which required a double crossover event to allow the insertion of novel DNA flanked by two regions of sequence homology. Homology-facilitated illegitimate recombination, where a single region of high sequence similarity initiates recombination that extends into areas of low sequence similarity, can also occur, although this generally happens at frequencies several orders of magnitude lower than for double-crossover events (1). However, since our PCR analysis of antibiotic-resistant colonies used primers that annealed to the homologous flanking region surrounding the insert, we were able to confirm that the entire non-homologous region was inserted into the genome at the correct locus, strongly suggesting a double-crossover event. We observed recombination of naturally transformed DNA with as little as 96 bp of flanking homology. However, it is possible that the MEPS for *X. fastidiosa* is lower than 96bp, but that recombination with these plasmids occurred at frequencies below our limit of detection (approximately 10^{-8}). In comparison, the MEPS for *E. coli* is 23bp, for *R. solanacearum* approximately 50bp, and approximately 70bp for *B. subtilis* (13, 37, 38). We observed an exponential relationship between flanking region length and recombination efficiency for *X. fastidiosa* up to 1kb, after which recombination efficiency was insensitive to increases in flanking region length. As the *X. fastidiosa* species shares a conserved set of core genes as well as more diverse flexible genes (23), the sequence similarity provided by the conserved core genes could facilitate recombination of more variable genes responsible for host adaptation or pathogenicity. However, unlike with sequence similarity and recombination efficiency, there is no consistent relationship between homologous flanking region length and recombination efficiency among different organisms. In *R. solanacearum*, for example, there appears to be a logarithmic relationship between homologous flanking region length and recombination efficiency, while in *B. subtilis* and *S. cereviseae*, the relationship is linear over the dynamic range (15, 37, 38). The differences among organisms could be based on the recombination pathway used or the efficiency of RecA, which detects homology between the donor and recipient DNA (13).

Our experimental data also provide a sense of how much non-homologous DNA can be inserted into the *X. fastidiosa* genome, which is relevant for assessing the potential of natural transformation and recombination to affect, for example, adaptation to novel environments. Previous work in *X. fastidiosa* has shown that altering a single gene can alter pathogenicity (24), illustrating how the insertion or replacement of as little as 1kb of DNA can significantly affect phenotype. In *R. solanacearum*, recombination efficiencies decreased exponentially with the

length of integrated non-homologous DNA (16). A similar relationship was observed in *X. fastidiosa*. No insertion of DNA longer than 4kb was observed, but it is possible that the integration of longer fragments occurred at frequencies below our limit of detection. During the transformation process, donor DNA is typically fragmented and reduced to single-stranded DNA before entering the cytoplasm (39). Fragmentation of plasmids with long stretches of non-homologous DNA is more likely to result in pieces containing only one homologous region, reducing recombination efficiencies. Likewise, for plasmids with long homologous flanking regions, fragmentation could trim the flanking region, possibly explaining why there was no difference in recombination efficiency for plasmids with 1kb to 4kb of flanking homology. Although our experiments only tested for recombination at a single locus in any given cell, studies with other naturally competent organisms have illustrated that an individual cell is capable of acquiring multiple independent strands of DNA at different loci by recombination (31, 40). It is possible that *X. fastidiosa* is also capable of such multiple cases of horizontal gene acquisition.

The analysis presented here illustrates the DNA size requirements necessary for efficient transformation and recombination in *X. fastidiosa*. We have also paired experimental data with *in silico* genome analysis to sample the effects of recombination on *X. fastidiosa* in natural environments. There is evidence that isolates of *X. fastidiosa* have been transferred between geographic regions (20, 23), and it has been hypothesized that the strain responsible for causing Pierce's disease in the United States diverged from an isolate introduced from Costa Rica (34). Understanding the DNA requirements for recombination could provide insights to how natural transformation affects the evolution of *X. fastidiosa* and other naturally competent bacteria.

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Table 1 Primers used to construct plasmids

Homology length		Insert Length	
Name	Sequence 5'-3'	Name	Sequence 5'-3'
26fwd	CATGAGCTCCGTATCAGGTCACAA	F1 fwd *Sph*I	ATAGCATGCCAGGTGTTCGATCC
26rev	CCGGAGCTCTACCATTACGGAGA	F1 rev *Nco*I	AACCATGGACGGGCTGTCTCTTATAC
35fwd	TGTGAGCTCTCCTTACGGCGTATC	F2 fwd *Sal*I	TAAGTCGACGTACAGCGGACATTTATTG
35rev	ATAGAGCTCCGACCGGACTACCAT	F2 rev *Sac*I	CGAGAGCTCCCTGGTACATCAGTC
50fwd	ATCGAGCTCAATAATGCTTCACGC	Insert rev *Nco*I	ATCCCATGGTAGAACAACCATTTATCG
50rev	AAAGAGCTCCGTCCGCAACAT	1k fwd *Not*I	TATAGCGGCCGCATGACAGTCCATGAAG
96fwd	TAAGAGCTCAGCATGGAACGCATA	2k fwd *Not*I	TATAGCGGCCGCTCTATTGATGGCTAGG
96rev	GCAGAGCTCGCACATAGAATCAAGt	3k fwd *Not*I	TATAGCGGCCGCTCTGCGGATAAAGGTA
200fwd	TACGAGCTCCCTTCTTCAGCTACG	5k fwd *Not*I	TATAGCGGCCGCCAGTGATGGTGG
200rev	ATAGAGCTCGACCGCCCTATTCC		
508fwd	ACAGAGCTCGTTCGGTGATGC		
508rev	GAAGAGCTCAATGCAGTGACGC		
760fwd	ATTGAGCTCTGTGGTGGTAAAGCG		
760rev	ATAGAGCTCGTATCCCAGATTGGCA		
1kfwd	AAAGAGCTCCAGGTGTTCGATCC		
1krev	CGAGAGCTCCCTGGTACATCAGTC		
2kfwd	ATAGAGCTCCTCCTTGAAGGAGGTGA		
2krev	TTAGAGCTCAGTGTGGCACCACTTC		
4kfwd	ATTGAGCTCTCAACCTATGCTGCCT		
4krev	ATAGAGCTCCAACGCCAAGAACAC		

Fig. 1

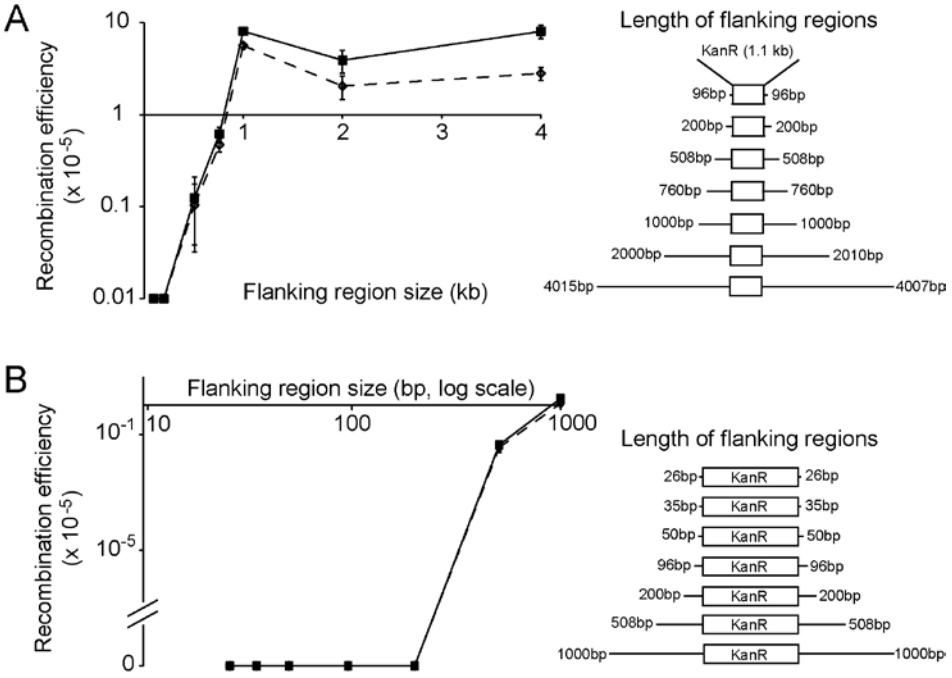

Fig. 1. Recombination efficiencies for plasmids with flanking regions varying from (A) 96 to 4000bp and (B) 26 to 1000 bp (note x-axis for B is on a log scale). Solid lines and squares show the recombination efficiencies (recombinants per total number of cells present) after normalizing for the total number of transforming DNA units present. Dotted lines and empty diamonds show overall recombination efficiencies. Recombination efficiencies peaked with approximately 1000bp of flanking region and then plateaued. Recombinants were recovered with as few as 96 bp of homology, but rates were essentially 0. Images on the right depict the regions cloned in to vector backbone pGEM-5zf(+) to create plasmids with various flanking regions. Homologous DNA was amplified from the *rpfF* region of *X. fastidiosa*.

Fig. 2

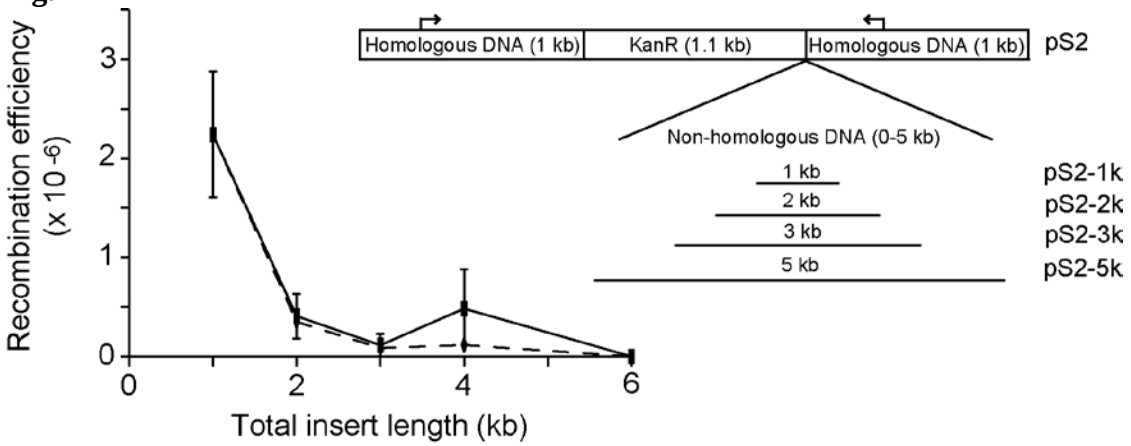

Fig. 2. Recombination efficiencies for plasmids with different lengths of non-homologous inserts ranging from 1kb to 6kb. Solid lines and squares show the recombination efficiencies (recombinants per total number of cells present) after normalizing for the total number of transforming DNA units present. Dotted lines and empty diamonds show overall recombination efficiencies. No recombinants were recovered when the total insert length was 6kb. The image on the right depicts the region cloned in to vector backbone pGEM-5zf(+) to create the plasmids. Flanking region was kept constant at approximately 1kb on either side. The non-homologous insert region consisted of a kanamycin cassette (approximately 1.1kb) and 0-5kb of DNA amplified from a plant virus with no regions of homology to the *X. fastidiosa* genome (total insert size for pS2 is approximately 1kb, for pS2-1k approximately 2kb, etc). Arrows over the homologous DNA regions show the location of primers rpfF-fwd and rpfF-rev used to screen antibiotic-resistant colonies for proper insertion of the kanamycin cassette and non-homologous DNA.

Chapter 4: Biological and genetic factors regulating natural competence in a bacterial plant pathogen

ABSTRACT

For naturally competent bacteria, biofilms can provide an environment for enhanced horizontal gene transfer through transformation and recombination. DNA is often an integral part of the extracellular matrix, 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 to liquid media, with maximum recombination efficiencies of about 10^{-3}. Cells were highly competent throughout their exponential growth phase, with no significant change in transformation and recombination efficiencies until the population ceased active growth. Mutations in type IV pili, competency-related and cell-cell signaling genes significantly impacted the ability of *X. fastidiosa* to acquire and incorporate DNA. As *X. fastidiosa* is highly competent when growing in a surface-attached state, as it does within insect vectors and host plants, transformation and recombination could be a significant route of horizontal gene transfer in its natural environments.

INTRODUCTION

In natural environments, bacteria often exist as biofilms, populations of matrix-enclosed bacteria that are generally adhered to a surface or interface (1). In many biofilms, the matrix that binds the cells together accounts for over 90% of the total mass, most of which is secreted by the cells themselves. The matrix is composed of extracellular polymeric substances, including polysaccharides, proteins, lipids and nucleic acids, with DNA often being a major component (2). In *Pseudomonas aeruginosa*, for example, extracellular DNA is required for biofilm formation, and the addition of DNase can dissolve immature biofilms (3). The needed extracellular DNA may result from cell lysis, although some bacteria actively secrete DNA through type IV secretion systems or membrane vesicles (4, 5). It has been estimated that the lysis of a single cell can increase local concentrations of DNA to above 100 µg/ml (6). High cell density and DNA concentrations make biofilms ideal environments for horizontal gene transfer to occur through natural transformation. In fact, it has been shown that horizontal gene transfer in general occurs at elevated rates in biofilm environments (7–9).

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 type IV pilus or type IV pilus-like apparatus and a series of competence-related proteins (encoded by *com* genes) for DNA uptake (5, 10, 11). Type IV pili may help stabilize the extracellular matrix of biofilms (2) and have been implicated in biofilm formation (12). Several *pil* genes are involved in the function and structure of these type IV pili. In *P. aeruginosa, pilQ* gene encodes an outer membrane multimeric protein that acts as the export pore for pili, while *pilO* likely encodes a cytoplasmic membrane-anchoring domain. PilB is a nucleotide binding protein necessary for the assembly of pili (14). A correlation has been shown between competence and the presence of pili, but it is unclear if pili play a direct role in transporting DNA across the membrane (10). Once transforming DNA crosses the outer membrane, its transport through the periplasm and inner

membrane is typically mediated by a series of *com* genes (10). 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 (10, 15).

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 (16). 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 (17–19). 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 (20).

Xylella fastidiosa is a vector-transmitted generalist plant pathogen that was recently found to be naturally competent and able to homologously recombine acquired DNA into its genome (21). It is often found in natural environments as an endophyte, but causes disease when it multiplies to high populations inside the xylem of its hosts. There, it inhibits the flow of xylem sap, leading to symptoms such as leaf scorching and stunted growth. *Xylella 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 (22). 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 sap, along with a smaller set of flexible genes that likely varies between strains and subspecies (23). Recent multi-locus sequence typing (MLST) of this species has shown that recombination plays a significant role in generating genetic diversity (24–26). In addition, some analyses suggest that recently emerged pathogenic strains of *X. fastidiosa* contain genetic elements potentially acquired from strains introduced from different geographic regions or infecting different host plants (23, 25, 27).

In natural environments, *X. fastidiosa* primarily lives in a surface-attached state, attached to either the xylem vessels of its host plants or its insect vectors' foreguts (22). Because of the linkage between biofilms and horizontal gene transfer, we assessed the effects of biofilm environments on the transformation and recombination efficiencies of this naturally competent pathogen as all previous studies of this phenomenon were conducted with planktonic cells in a defined-nutrient liquid medium (21, 28). We also determined the extent to which several *com*, *pil*, and other genes contribute to *X. fastidiosa*'s ability to acquire and recombine DNA.

MATERIALS AND METHODS

Strains, plasmids, media, and growth conditions. *Xyella fastidiosa* subspecies *fastidiosa* strain Temecula (29) 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 (30). A *pglA* mutant (strain Fetzer), in which the polygalacturonase gene has been disrupted by a kanamycin resistance cassette, was obtained from Roper et al. (31). All other mutants, which are kanamycin-resistant, were either generated in this study or obtained from other sources (Table 1). Plasmids p1k (28) 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 (21, 32). Expression of the methylase was induced by supplementing the growth

medium with 1mM IPTG. *Xyella fastidiosa* cells were propagated on periwinkle wilt medium with (PWG) or without Gelrite (PW) (33) or on *X. fastidiosa* minimal medium (XFM). We used liquid XFM as previously described (21), and added 10g/L of Gelrite and increased $MgSO_4$ to 1g/L (Gelrite does not properly solidify with only 0.5 g/L) for solidified medium. When appropriate, kanamycin was added to a final concentration of 30 µg/ml and chloramphenicol was added to a final concentration of 10 µg/ml. Cells were incubated at 28°C with shaking at 180 rpm when appropriate.

General transformation protocol. Transformation in liquid culture was done as previously described (21). 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 two days of growth at 28°C with shaking, DNA was added to a final concentration of 5 µg/ml. Cells were plated on selective media after an additional 24 hours 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 µl 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 plasmid in approximately 10 µl was spotted directly on top of the cells. After a 24 hour incubation period, cells were suspended in 250 µl buffer (50mM NaCl and 10mM 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 (21). A portion of the culture from each transformation experiment was also saved for quantification by qPCR as previously described to determine recombination efficiency, defined as the number of recombinants divided by the number of cells present (21).

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 two days of growth. After an additional 24 hour incubation period, cells were suspended and plated on PWG with kanamycin. This experiment was repeated, each time with 12 replicates for each treatment. Data from the two experiments were combined after determining that they 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=.054 for PWG and XFM respectively).

To test the effect of the initial culture medium 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 for liquid media with p1k and 18 replicates for each 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) were co-cultured on solid XFM to compare 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 one hour 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. A random sample of doubly-antibiotic resistant colonies were

screened with PCR using primers NS1-f/r and pglA-fwd/rev (21) to confirm the presence of both antibiotic cassettes in the correct loci.

Population growth experiment. To study the effects of population growth stage on the competence of cells in biofilms, 10 µl samples of *X. fastidiosa* cell suspensions at OD_{600}=0.25 were spotted on solid XFM plates and incubated at 28^{o}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). In addition, to measure recombination efficiency, six 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 approximately 10 µl volume directly on top of the cells, incubating for an additional 24 hours, and dilution plating on PWG with kanamycin. Aliquots were also saved for qPCR and 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 5 µM SYTO 9 and 30 µM propidium iodide to 50µl of cell suspension and incubated the mixture for approximately 15 minutes in the dark at room temperature. Fluorescence was viewed with a Zeiss AxioImager M1 with Chroma (Bellows Falls, VT) 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 PCR reactions for cloning purposes utilized Finnzymes Phusion Hot Start Polymerase (Thermo Fisher Scientific, Lafayette, CO). Regions of between 300 and 1000 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, Madison, WI) to create pF1F2. The kanamycin cassette from pAX1-Km (30) 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 two 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 (30). 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 and efficiencies were log-transformed for statistical analysis to fit model assumptions of homogenous variance. Log-transformed efficiencies were subjected to an analysis of variance 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'

(34). Differences between mutants were determined by Tukey's HSD test using the 'glht' function in package 'multcomp' (35). Antibiotic-resistant colonies for each mutant were randomly selected for screening by PCR using primers NS1-f and NS1-r (21) 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 (28), a plasmid containing a kanamycin cassette flanked on either side by approximately 1kb 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). Recombination efficiency on solid XFM was significantly higher than on solid PWG (t_{23df}= 11.98, p= 2.29×10^{-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. The order in which the cells 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 medium decreased over time (21). To determine if this decrease was an effect of cells transitioning from a solid-state to planktonic phenotype, recombination efficiencies in liquid XFM were measured for cultures inoculated with cells harvested from solid PWG or XFM plates (Fig. 2). 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 statistically significant (t_{22df}= 4.18, p=0.0004).

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 biofilm environment 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 7 days after inoculation (r^2= 0.89, p< 1×10^{-11}), and 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. Similar results were seen in a replicate experiment without live/dead staining of cells (data not shown). In addition, competence was positively correlated with the rate of change of the cell population (Fig. 3B). 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 ($y= 0.0632x-8.38$, $r^2= 0.914$, $p= 3.97x10^{-26}$).

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 4 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*) or previous microarray data indicating upregulation when grown in liquid XFM compared to liquid PW (*traD*, putative conjugal transfer protein, and *ssb*, single-stranded DNA binding protein) (36), as cells are more competent in XFM. Type IV pili and a series of *com* genes are necessary for efficient transformation in a range of naturally competent bacteria (5, 10, 11), and previous work has suggested that cell-cell signaling may affect competence in *X. fastidiosa* (21). A variety of other strains with mutations in genes not expected to affect competence were selected as controls. The *fimA, rpfC, ssb, hxfB,* and *chiA* mutants exhibited recombination efficiencies that were statistically equivalent to the wild type ($p>0.05$). 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.

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 medium; in comparison, transformation efficiency of the same plasmid in liquid medium is only about 10^{-5} (28). Although the total amount of plasmid added to cells grown on solid medium was kept constant, biofilms are heterogeneous environments and thus we were unable to control local concentrations of DNA or ensure that the plasmid was evenly distributed among cells. However, because of different population sizes, the ratio of DNA to cells was approximately 10 times higher for cells growing in liquid compared to solid media, so DNA concentration was not likely a dominant factor causing an increase in recombination efficiency.

We observed similarly high recombination efficiencies between different antibiotic resistant strains of *X. fastidiosa* when co-cultured on solid medium. 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 of same two strains co-cultured in liquid XFM (21). As in the previous study, it is quite likely that actual recombination rates between the strains were higher than observed, as any recombination events that did not involve the transfer of one of two marker genes would not have been detected.

We were also able to transform *X. fastidiosa* on solid PWG medium, but the process was about 100-fold less efficient than on solid XFM. It was originally reported that *X. fastidiosa* was not competent when grown in liquid PW (21). It is possible that cells are naturally competent in liquid PW but they acquire DNA at such a low frequency that events are difficult to detect in the smaller population sizes achieved in liquid media. The decline in competence observed in high nutrient environments (PWG versus XFM) supports the hypothesis that competence originally developed as a nutrient uptake system (16).

Based on the difference in competence between biofilm and planktonic cells, we hypothesized that the previously observed decrease in recombination efficiency over time in

liquid medium after inoculation with plate-grown cells (21) reflected the transitioning of highly competent biofilm cells in the inoculum to less competent planktonic cells. However, results do not support this model. The relatively small difference observed in recombination rates for cells harvested from PWG and XFM and transformed in liquid medium (1.57×10^{-6} and 5.58×10^{-6}) suggests that the phenotype of the initial inoculum has little effect on subsequent competence.

As population growth was likely the dominant factor linked to decreased recombination in broth culture (21), we assessed growth effects on biofilms cells on plates. Overall, competence was linked directly with the rate of population growth. Recombination efficiencies remained relatively constant as the population underwent exponential growth. Recombination efficiencies then dropped by several orders of magnitude as the population entered stationary and death phase. This drop was most likely associated with the change in the rate of population growth instead of, for example, an increase in competing DNA available for transformation supplied by dead cells. After populations of live and dead cell stabilized around day 14, and the amount of DNA present from dead cells no longer increased, recombination efficiencies continued to drop. Thus, as with planktonic cells, transformation and recombination for surface-attached *X. fastidiosa* appears tightly correlated with exponential growth. In *Ralstonia solancearum*, to our knowledge the only other documented naturally competent plant pathogen, there is a similar correlation between competence and exponential growth (37).

In natural environments, donor DNA for transformation can be supplied by the lysis of dead cells or actively secreted from living cells (5, 38). In our studies, 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 neighboring (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 previous work in liquid medium has shown that planktonic cells increase extracellular DNA in the growth medium over time and can be transformed with as little as a few picograms per microliter of extracellular DNA (21). There is also evidence that DNA is necessary for efficient biofilm formation in *X. fastidiosa* (39). The lysis of a single cell in a biofilm can result in local concentrations of DNA of up to 100 µg/ml (6). Thus, even if the number of dead donor cells available to provide extracellular DNA is relatively low during periods of rapid growth associated with competence, there could still be sufficient DNA available for transformation, especially within biofilms. 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 (37). Perhaps *X. fastidiosa* cells are mechanically lysed during the vector transmission process, providing additional extracellular DNA. Alternatively, *X. fastidiosa* may actively export DNA as observed in other taxa, such as *Neisseria gonorrhoeae* (5), although no evidence has been found suggesting this is the case.

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*. Performing the experiments on solid media enabled detection of low-probability events in certain mutant backgrounds. Populations also remained competent for longer periods of time on solid media. When grown in liquid XFM, *X. fastidiosa* competence peaked after two days of growth and then rapidly declined with time (21); on solid XFM, transformation and recombination efficiencies remained relatively high and constant for at least five days after inoculation. Thus, any differences in growth rates between the wild type and mutant strains would be unlikely to affect transformation and

recombination efficiencies. As expected, disruption of several genes not presumed to be involved in DNA uptake (type I pilus protein *fimA*, chitinase *chiA* and hemagluttinin adhesion *hxfB*) had no effect on competence. Disruption of the *ssb* gene, 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, so disrupting one copy would be unlikely to affect the cell's ability to recombine acquired DNA. The *tolC* mutant, however, was not transformable. TolC is one of three component proteins comprising the multidrug resistance efflux pump (40). In Gram-negative bacteria, TolC traverses the periplasm and outer membrane. It is possible that a loss of TolC could affect DNA uptake across the outer membrane, even if it is not directly involved in DNA transport. The putative conjugal transfer protein *traD* had slightly lower recombination efficiencies than the wild type. Even though conjugation and transformation are distinct processes, it is possible that DNA transport across the membrane is also impacted in this mutant.

Transformation and recombination efficiencies were lower in all four type IV pili mutants tested. In *X. fastidiosa*, type IV pili are also involved in twitching motility (41). Two of the type IV pili mutants tested were transformable, but at efficiencies at least 10-fold lower than the wild type. Disrupting the *pilY1* gene had the least pronounced effect on competence, with efficiencies about 7% of the wild type. The *pilY1* gene is thought to be involved in cell adhesion and is localized at the pilus tip (13, 42). In *X. fastidiosa*, *pilY1* mutants still produce type IV pili that appear similar to wild type pili, but the mutants have reduced twitching motility (13). Thus, it is possible that in the *pilY1* mutant the necessary structure for DNA transport through the membranes is still intact. The other three *pil* mutants we tested either had markedly lower recombination efficiencies (approximately 0.35% of wild type for the *pilB* mutant) or were untransformable (*pilO* and *pilQ* mutants). Previous work has shown that these three *pil* mutants do not exhibit any twitching motility. In addition, transmission electron microscopy showed that the *pilB* mutant and several other *pil* mutants that do not exhibit twitching motility do not produce any type IV pili (41). Based on homology to the *pil* genes in *P. aeruginosa* and the lack of twitching motility (13), the PilB, PilO and PilQ proteins in *X. fastidiosa* are likely required for pilus assembly and retraction; a mutation in any of these genes 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. Our results suggest DNA may be able to pass through the inner membrane channel passively, but at only about 1% the rate of passage in wild type cells. The decreased recombination observed in the type IV pili and *com* mutants suggest that DNA transport from the extracellular environment into the cytoplasm in *X. fastidiosa* 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* genes.

Significantly lower transformation and recombination efficiencies were observed in an *rpfF* mutant (approximately 0.15% of wild type efficiencies), but an *rpfC* mutant recombined at the same rate as the wild type. Cell-cell signaling in *X. fastidiosa* is mediated by the small molecule *D*iffusible *S*ignaling *F*actor (DSF); the *rpfF* gene encodes a DSF synthase, while *rpfC* encodes a two-component regulator that senses DSF. In *X. fastidiosa*, *rpfF* mutants do not produce DSF, while *rpfC* mutants overproduce DSF (43). In *rpfC* mutants, *rpfF* is overexpressed when compared to wild type, and the two mutants essentially exhibit opposite phenotypes for traits controlled by genes regulated by *rpfF* and *rpfC* (44). 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 *Vibrio cholerae* (45). 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 quorum sensing. It is also interesting to note that in in PW, *rpfF* mutants do not form biofilms as readily as the wild type (46).

Increased rates of horizontal gene transfer have been observed between bacteria living in biofilm environments (7, 9). For naturally competent bacteria, transformation is likely a prominent mode of this horizontal gene transfer in biofilms (8, 15). Extracellular DNA is a major component of the biofilm matrix for many organisms, and there is evidence that it is required for efficient biofilm formation in *X. fastidiosa* (39). 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 in natural environments (21, 22), 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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Table 1. Mutant strains used in this study.

	Locus	Name	Putative Function	Pfam Analysis	Recomb. Eff	Source
com genes	0042	*comF*	competence protein F	Phosporibosyl transferase 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	-	(13)
	0062	*fimA*	type I pilus protein, surface attachment and biofilm formation	n/a	=	(41)
	1691	*pilQ*	type IV fimbrial biogenesis outer-membrane protein	STN family, Secretin N domain, Secretin family	-	(41)
	1693	*pilO*	type IV fimbrial biogenesis cytoplasmic-membrane protein	PilO family	-	(13)
	1927	*pilB*	type IV fimbrial biogenesis cytoplasmic ATPase	TS2E Nter domain, Type II/IV ss protein family	-	(41)
cell-cell signaling	0406	*rpfC*	autoregulator of DSF biosynthesis	Response regulator domain, Histidine kinase domain	=	(21)
	0407	*rpfF*	DSF biosynthesis gene	Enoyl CoA hydratase/isomerase family	-	(43)
other	0952	*traD*	conjugal transfer protein	NA	-	This study
	1017	*ssb*	ssDNA binding protein	Single-stranded DNA binding family domain	=	This study
	1792	*hxfB*	hemagluttinin adhesin	hemagluttinin repeats, hemagluttinin activity domain, extended signal peptide of Type V secretion system	=	(47)
	1826	*chiA*	chitinase	Glycosyl hydrolase family	=	Labroussaa and Almeida, unpub.
	1964	*tolC*	multidrug resistance efflux pump, outer membrane export factor	Outer membrane efflux protein	-	(40)

Table 2. Primers used in this study.

Function	Name	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.

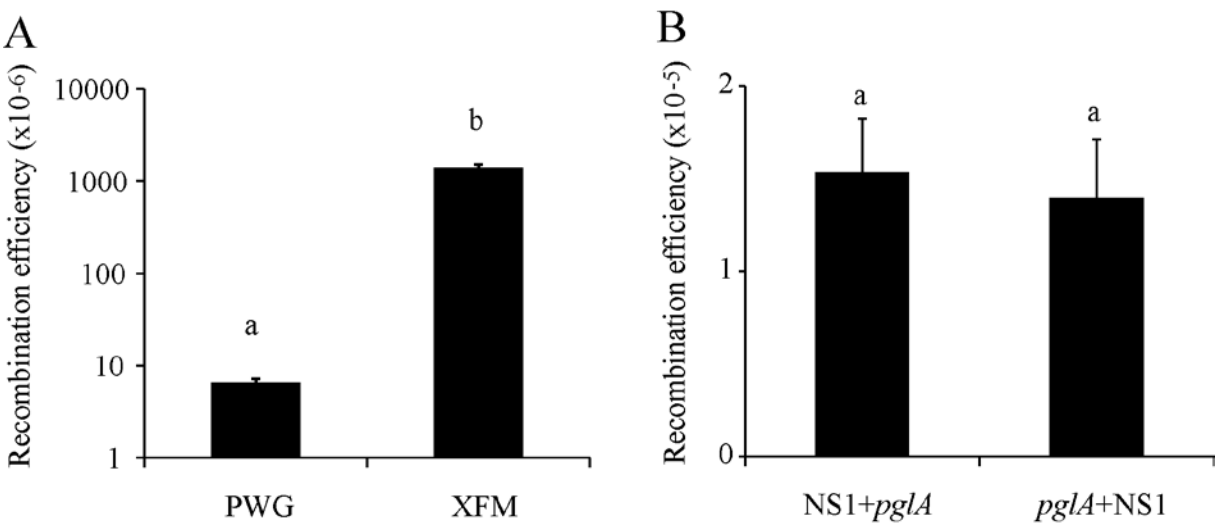

Fig. 1. Recombination efficiency of cells on solid media. (A) Recombination efficiencies of strain Temecula transformed with p1k. Rates of 6.5×10^{-6} were observed for cells grown on PWG and 1.4×10^{-3} for cells grown on XFM. (B) Efficiency of marker gene transfer between different strains of *X. fastidiosa* spotted on top of each other on solid XFM. Aliquots were allowed to dry for approximately 1 hour before the second strain was spotted directly on top of the first. The order in which the cells were spotted on the plate did not significantly affect recombination efficiencies. Letters indicate statistically different treatments.

Fig. 2.

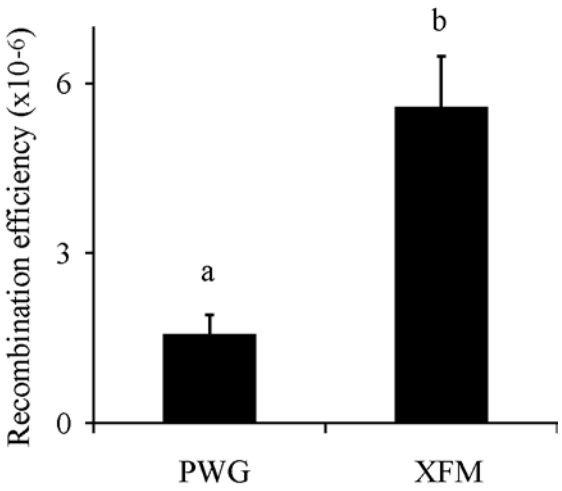

Fig. 2. Effect of initial inoculum on competence in liquid XFM. Cells were grown for approximately 1 week on solid PWG or XFM before being diluted to OD_{600} of 0.01 in liquid XFM. Cultures were then grown for 2 days before adding 5ug/ml of p1k. The average recombination efficiency for cells initially grown on PWG was 1.57×10^{-6}, and 5.58×10^{-6} for cells initially grown on XFM. Different letters indicate statistically different treatments.

Figure 3.

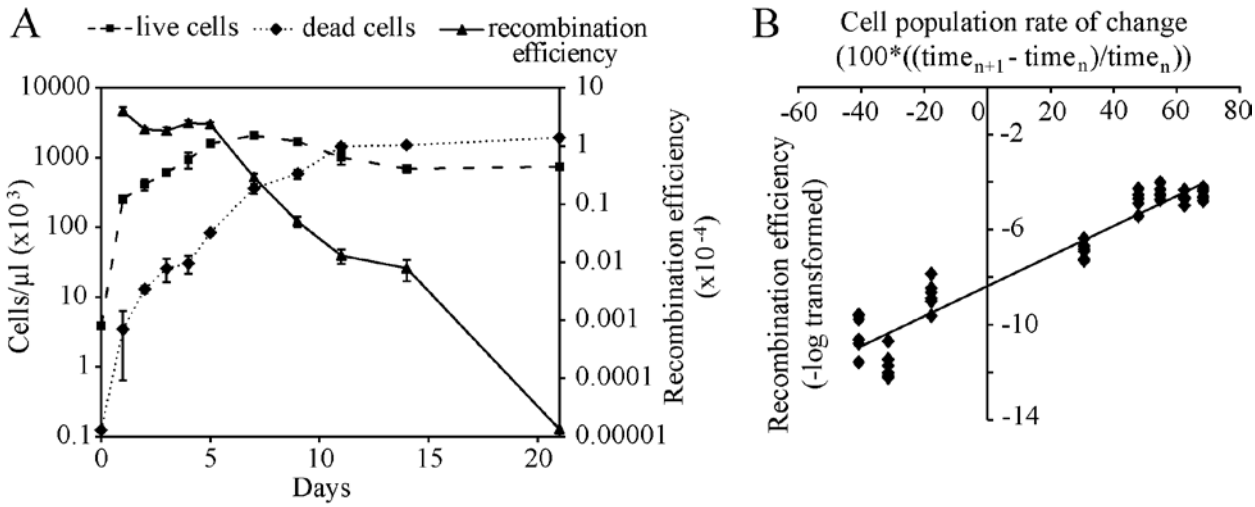

Fig. 3. Competence and growth of *X. fastidiosa* on solid XFM over time. (A) Live (squares, dashed line) and dead cell populations (diamonds, dotted line) were quantified using qPCR and SYTO 9/ propidium iodide staining over the course of 21 days. Cells grew exponentially with over 95% of the population consisting of live cells for the first five days; after day seven, the live cell population began to decrease and by day 11, more dead cells than live cells were present. 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. Similar results indicating a drop in recombination efficiency as total cell population plateaued were observed in a separate experiment (data not shown). (B) A correlation was found between the negative log-transformed recombination efficiencies and the rate of change of the population ($y= 0.0632x-8.38$, $r^2=0.914$, $p=3.97x10^{-26}$). Data from day 1 and day 21 were outliers and removed from the analysis.

Fig. 4.

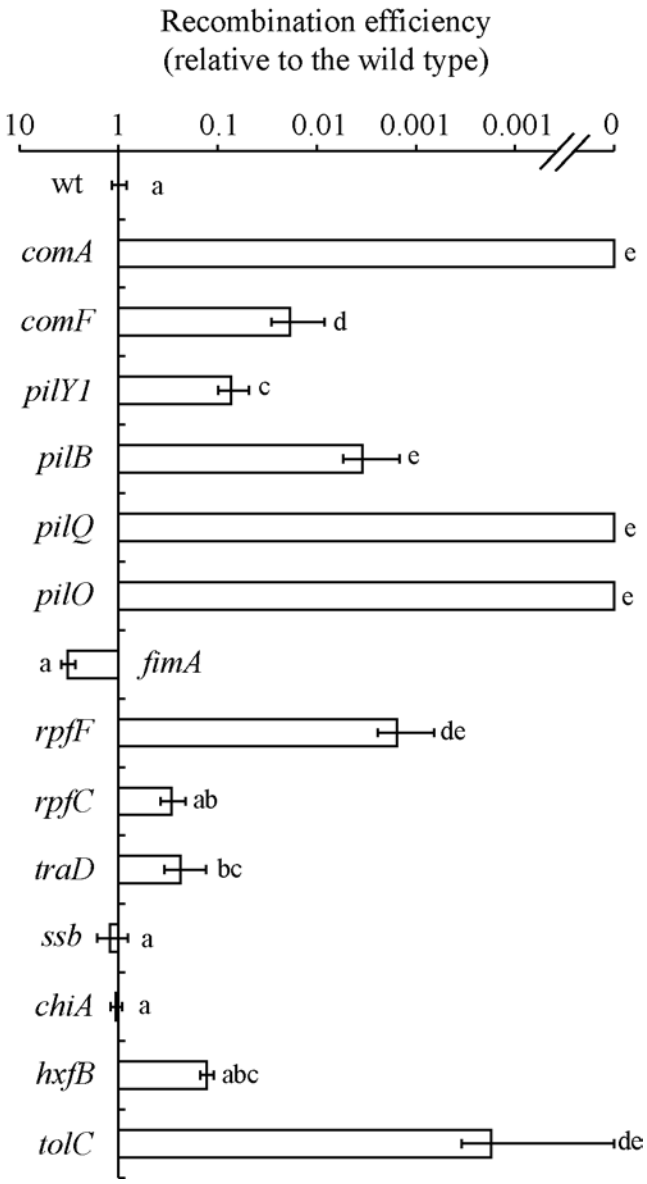

Fig. 4. Relative recombination efficiencies of *X. fastidiosa* mutants compared to the wild type (wt). Letters indicate statistically different groups ($p<0.05$). All *com* and type IV pili gene mutants tested had significantly lower recombination efficiencies than the wild type, as did the *rpfF*, *tolC* and *traD* mutants. The *fimA*, *rpfC*, *ssb*, *chiA* and *hxfB* mutants all had transformation and recombination efficiencies statistically equivalent to the wild type.