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Natural Competence of *Xylella fastidiosa* Occurs at a High Frequency Inside Microfluidic Chambers Mimicking the Bacterium's Natural Habitats

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ABSTRACT

Xylella fastidiosa is a xylem-limited bacterium that is the causal agent of emerging diseases in a number of economically important crops. Genetic diversity studies have demonstrated homologous recombination occurring among *X. fastidiosa* strains, which has been proposed to contribute to host plant shifts. Moreover, experimental evidence confirmed that *X. fastidiosa* is naturally competent for recombination *in vitro*. Here, as an approximation of natural habitats (plant xylem vessels and insect mouthparts), recombination was studied in microfluidic chambers (MCs) filled with media amended with grapevine xylem sap. First, different media were screened for recombination in solid agar plates using a pair of *X. fastidiosa* strains that were previously reported to recombine in coculture. The highest frequency of recombination was obtained with PD3 medium, compared to those with the other two media (*X. fastidiosa* medium [XFM] and periwinkle wilt [PW] medium) used in previous studies. Dissection of the media components led to the identification of bovine serum albumin as an inhibitor of recombination that was correlated to its previously known effect on inhibition of twitching motility. When recombination was performed in liquid culture, the frequencies were significantly higher under flow conditions (MCs) than under batch conditions (test tubes). The recombination frequencies in MCs and agar plates were not significantly different from each other. Grapevine xylem sap from both susceptible and tolerant varieties allowed high recombination frequency in MCs when mixed with PD3. These results suggest that *X. fastidiosa* has the ability to be naturally competent in the natural growth environment of liquid flow, and this phenomenon could have implications in *X. fastidiosa* environmental adaptation.

IMPORTANCE

Xylella fastidiosa is a plant pathogen that lives inside xylem vessels (where water and nutrients are transported inside the plant) and the mouthparts of insect vectors. This bacterium causes emerging diseases in various crops worldwide, including recent outbreaks in Europe. The mechanisms by which this bacterium adapts to new hosts is not understood, but it was previously shown that it is naturally competent, meaning that it can take up DNA from the environment and incorporate it into its genome (recombination). In this study, we show that the frequency of recombination is highest when the bacterium is grown under flow conditions in microfluidic chambers modeled after its natural habitats, and recombination was still high when the medium was amended with grapevine sap. Our results suggest that this bacterium is able to recombine when growing inside plants or insects, and this can be a mechanism of adaptation of this pathogen that causes incurable diseases.

Natural competence is a phenomenon that allows bacteria to take up DNA segments from the environment and incorporate them into the genome via homologous recombination (1). Natural competence was first demonstrated in *Streptococcus pneumoniae* in 1928 by Frederick Griffith (2). Griffith showed that virulence genes were transferred from donor to recipient cells, converting the nonvirulent recipients into virulent pathogens (2). Since then, ~80 bacterial species in divergent phyla have been described as naturally competent (3). Although the exact reasons for occurrence of natural competence in bacteria still remain unknown, studies showed that natural competence is induced under conditions of starvation (4–6) and DNA damage (7), and it has been hypothesized that the incoming DNA serves as a food source and DNA repair material. Another proposition is that natural competence allows acquisition of new genes and alleles, providing the recipient cells with adaptive advantages. In fact, a previous study showed an increased rate of adaptation by natural competence in *Helicobacter pylori* (8). Interestingly, natural competence has been demonstrated in some of the most highly diverse and

successful human pathogens such as *H. pylori* (9, 10), *Neisseria meningitidis* and *Neisseria gonorrhoeae* (11, 12), and *Porphyromonas gingivalis* (13), which require rapid adaptation to evade the immune response. Furthermore, natural competence also was described in two plant pathogens, *Ralstonia solanacearum* (14) and

Xylella fastidiosa (15), both of which have very broad plant host ranges.

Xylella fastidiosa is a bacterial pathogen affecting many economically important crops, such as grape, citrus, coffee, peach, and almond (16). The disease process is not completely understood, but it is proposed that *X. fastidiosa* forms biofilm-like aggregates and blocks xylem vessels, the conduits for water and nutrient transport in the plants (17). This blockage hinders xylem sap flow and starves the upper aerial parts of water and mineral nutrients, producing symptoms that resemble those of water and nutrient deficits. *X. fastidiosa* is transmitted by a number of xylem sap-feeding insects, including sharpshooter leaf hoppers and spittlebugs in which *X. fastidiosa* forms biofilms in the foregut (18, 19). Taxonomically, *X. fastidiosa* is divided into five subspecies based on multilocus sequence typing (MLST) (20, 21). Even within the subspecies, host range and genotype diversity have been described (22, 23), and recombination events among strains have been detected among field-collected samples (22, 24). In fact, homologous recombination was shown to have a greater effect in generating genetic diversity in *X. fastidiosa* than point mutation (20). Recent outbreaks of *X. fastidiosa* diseases in Europe (25) and Asia (26, 27) and also in new plant hosts such as olive (25), blueberry (28), and pear (26) suggest the great adaptation potential of this pathogen.

In a number of plant species, *X. fastidiosa* is believed to live as a harmless endophyte without inducing disease symptoms (17, 29). Coexistence in the same xylem system of different strains for a long time without killing the host represents a fertile environment for exchange of DNA material. Several MLST-based studies detected intersubspecific recombination among strains of *X. fastidiosa* and proposed recombination as the mechanism of new allele acquisition, leading to plant host shift and disease emergence. Intersubspecific recombination was described to generate strains that infect citrus and coffee (30), mulberry (21), and blueberry and blackberry (31). A recent study also showed intersubspecific recombination between coffee-infecting strains in plants intercepted in France (32). Natural competence could be an explanation for the frequent recombination events detected in *X. fastidiosa*.

Natural competence in *X. fastidiosa* was recently described *in vitro* (15), the rate of homologous recombination was shown to be higher when the cells were growing exponentially in solid agar plates than in batch culture tubes, and minimal medium was more conducive than rich medium (33). With a plasmid as a donor DNA, 96 bp of flanking homology was sufficient to initiate recombination (34). Moreover, some competence-related and type IV pili genes were shown to be involved in the process (33). Although some of those studies were performed using plasmids as donor DNA, two strains were also shown to recombine in coculture conditions (15), although the capacity of these strains to act either as a donor or a recipient for DNA exchange was not determined in those studies.

The objective of this study was to test the hypothesis that natural competence in *X. fastidiosa* occurs under flow conditions (an approximation of the natural habitat of this bacterium). Associated with this objective was the aim of elucidating whether previous observations of high frequencies of *X. fastidiosa* natural competence *in vitro* (15, 33, 34) were dependent on batch culture conditions (both test tubes and agar plates), which allow cell-to-cell contact for longer times without replenishing of nutrients or

removal of secreted molecules. Although natural competence and recombination are assumed to occur in natural habitats based on field surveys and DNA sequence data, experimental indications of its occurrence in the plant or insect host are not yet available for *X. fastidiosa*. Therefore, to circumvent the limitation of *X. fastidiosa* recombination tests in the natural hosts that are affected by uneven bacterial distribution and low populations (29, 35), we performed recombination experiments in a microfluidic chamber (MC) system that mimics the natural environment of xylem vessels and insect foreguts. The MC system allows continuous media flow conditions and formation of biofilms and has been previously used to study the behavior of *X. fastidiosa* (36–39). The biofilm fraction of the MC (referred here as MC_in) and the planktonic and detached cell fraction (referred here as MC_out) can be collected separately, and the behavior of cells in the two fractions can be determined. Two strains used in all of the previous publications on this topic (15, 33) were used in this current study to facilitate comparison with the literature and to further our understanding of natural competence in *X. fastidiosa*. The results presented here show that growth under flow conditions supports natural competence in *X. fastidiosa*, with recombination frequencies equivalent to that on solid media, previously described to be the most conducive environment for natural competence *in vitro* (33). These findings support the hypothesis that recombination occurs at high rates under flow conditions, representing the natural habitats of *X. fastidiosa*.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *Xylella fastidiosa* subspecies *fastidiosa* mutants NS1-CmR (a mutant of the wild-type strain Temecula1 in which a chloramphenicol [Cm]-resistant cassette was inserted in a noncoding region using the suicide plasmid pAX1-Cm [40]) and *pglA*-KmR (a kanamycin [Km]-resistant mutant of the Fetzer strain in which the gene encoding polygalacturonase is disrupted [41]) were used in this study. The mutants were cultured in periwinkle wilt (PW) agar medium (42), modified by omitting phenol red and adding 1.8 g liter^{-1} bovine serum albumin (BSA) (Gibco Life Science Technology) and supplemented with the respective antibiotics. PD3 medium (43) and modified *X. fastidiosa* medium (XFM) (15) were used when stated. Pectin was added to a final concentration of 0.01% as previously described (44). Kanamycin was used at 30 μg ml^{-1} and chloramphenicol at 10 μg ml^{-1}. Inocula were prepared by streaking cultures from the −80°C freezer stocks on PW agar plates and incubating the plates for 5 to 7 days at 28°C. Cultures were then restreaked onto new plates and incubated for another 5 to 7 days before use.

Media selection for natural competence and growth in microfluidic chambers. To select a medium to test the occurrence of natural competence in MCs, three media (PW, modified XFM, and PD3) were first tested in solid agar plates. XFM and PW, used in previous studies (15, 33), were selected as positive- and negative-control media, respectively, for recombination. Natural competence experiments were performed according to the method of Kung et al. (15, 33) with some modifications. Briefly, cells (optical density at 600 nm [OD$_{600}$] of 0.25) of the NS1-CmR and *pglA*-KmR mutants were prepared in liquid media by scraping the cultures from PW-antibiotic plates. Ten microliters of each strain was spotted on top of each other on the agar plates of PW, XFM, and PD3 without antibiotics, and the spots were allowed to dry for ~1 h. The plates were then incubated at 28°C for ~3 days. Next, two spots from the same plate were scraped off and suspended in 1 ml of PD3 to make one replication, and 3 to 4 replicates were included for each media type per experiment. The experiments were repeated independently twice for XFM and at least three times for PD3 and PW. Single mutant strains (donor and recipient) were included as controls. The suspensions were then serially diluted, and

100-μl aliquots of appropriate dilutions were plated on PW agar plates in triplicate supplemented with both antibiotics (Km and Cm) to recover recombinants at the antibiotic-resistant site and with a single antibiotic (Km or Cm) to check for the growth of both parents in the mixture. Appropriate dilutions also were plated onto PW plates without antibiotics for enumeration of total viable cells. Plates were incubated at 28°C for at least 14 days before CFU were enumerated. The recombination frequency at the antibiotic-resistant site was calculated as the ratio of recombinant CFU (CFU from both antibiotic plates) to total CFU (CFU from appropriate no-antibiotic plates) in equal volumes of suspension. After selection of the media that supported recombination in the agar plates, the media (PD3 and XFM) were tested in the MCs for cell attachment and biofilm formation.

Media components influencing natural competence and twitching motility. To test for the specific components that may influence natural competence, an initial screen was performed by removing or adding components to PW and PD3 in solid agar plates as described above. The components tested were sodium citrate dehydrate, succinic acid, and starch (present only in PD3); BSA and L-glutamine (present only in PW); and pectin. The effect of BSA was further tested by supplementing PD3 and PD3 plus L-glutamine with BSA and removing BSA from PW and XFM. Experiments were repeated three times independently with three replicates each time, except for PD3 plus L-glutamine treatment that was performed once with three replicates. The twitching motilities of both mutants were determined in media with and without BSA, according to previous studies (45, 46) with few modifications. Briefly, for PD3 and PW with and without BSA, media plates solidified with agar or Gelrite (RPI Research Products International, Mount Prospect, IL) were divided into two halves, 10 to 12 spots of each mutant strain were made using a sterile toothpick, and plates were incubated at 28°C for 4 to 5 days. For XFM with and without BSA, plates solidified with agar were used and incubated for 10 to 12 days before measurements were recorded. Colony peripheral fringes were observed under ×10 magnification using a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY), and fringe widths were measured for six colonies per plate per strain, with at least seven measurements per colony using a Nikon DS-Q1 digital camera (Nikon) connected to a Nikon Eclipse Ti inverted microscope and controlled by NIS-Elements imaging software version 3.0. Twitching experiments were performed at least three times independently for PD3 and PW with and without BSA and once for XFM with and without BSA.

Natural competence under different growth conditions. Three growth conditions were used: solid agar plates (plates, as described above), liquid culture tubes (tubes), and continuous liquid flow (MCs). PD3 without antibiotics was the medium used, and the initial inocula of the NS1-CmR and pglA-KmR mutants were prepared as described above.

(i) Competence in tubes. Twenty-five-milliliter glass test tubes containing 3 ml of PD3 were inoculated with 100 μl of each of the OD-adjusted strain suspensions as donor and recipient cells (final OD_{600} of 0.01). Tubes containing single strain inoculations were included as control treatments. Tubes were then incubated with shaking (180 rpm at 28°C). After ~3 days, the tubes were vortexed well to mix the biofilm formed on the air-liquid interface with the rest of the suspension and serially diluted and plated as described above. Three independent experiments were performed, and three replications were included in each experiment ($n = 9$ in total).

(ii) Competence in MCs. MCs were prepared as previously described (36). Briefly, two parallel channels with separate inlets for bacterial cells and growing media were etched on a silicon wafer. The channels were modeled into polydimethylsiloxane (PDMS) and sandwiched between the PDMS layer and a glass cover slide. The inlets and outlets were then connected to tubings that were connected to syringes (see Fig. S1 in the supplemental material). The syringes were connected to pumps which control the media flow rate in the MC. The MC was mounted onto a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY) to observe cell attachment and biofilm formation using phase-contrast and No-

marski differential interference contrast (DIC) optics. Time-lapse video was taken using a Nikon DS-Q1 digital camera (Nikon) connected to the microscope and controlled by NIS-Elements imaging software version 3.0.

For preparing the inocula for MCs, equal volumes of the strain pairs (OD_{600} of 0.25) were mixed and inoculated into the cell inlet syringes, and growing medium (PD3) was injected in the media syringes. MCs were run for 5 to 7 days with a media flow rate of 0.25 μl min^{-1} until abundant growth of biofilm was observed. At the end of the experiment, the fraction of cells collected in the outlet syringe (MC_out) was harvested, and the fraction formed inside the channels (MC_in) was detached and pushed to the outlet collection syringe by increasing the flow rate to 30 to 40 μl min^{-1}. Serial dilution, plating, CFU counts, and the frequency of recombination calculations were done as described above. Four independent experiments were performed with seven replicates in total ($n = 7$).

(iii) Competence in MCs with grapevine sap. Grapevine (*Vitis vinifera*) sap was collected from a *X. fastidiosa*-susceptible variety (Chardonnay) in Dahlonega, GA, and a tolerant variety (Blanc Du Bois) in Tallahassee, FL, at the end of the dormant season (March/April). A new season cane was pruned, and sap was collected in a 50-ml conical tube, which was stored in ice until it was brought back to the lab. Xylem sap was sterilized by filtering with a 0.22-μm vacuum filter and stored at −80°C until used. Sap experiments were performed in the MCs with both pure sap and 50% sap mixed in PD3 (vol/vol). Natural competence assays were same as those for the MC experiment with PD3. Experiments were repeated at least three times for both sap types.

Natural competence with heat-killed donor cells and confirmation of homologous recombination. Confirmation of homologous recombination occurring via natural competence was performed by using heat-killed donor cells in the solid agar plates. Suspensions of the donor cells (either the NS1-CmR or the pglA-KmR mutant) were incubated at 90°C for 15 min for heat killing. Complete killing was confirmed by plating an aliquot onto PW plates. The heat-killed donor and live recipients were then spotted on PD3 plates as described above. For confirmation of homologous recombination at the desired genome region, randomly selected recombinant CFU were restreaked onto new double-antibiotic PW plates, and colony PCR was performed using the primers targeting the flanking region of the construct used to generate the mutants according to Kung et al. (15). Sequences of the flanking regions of antibiotic cassette insertion sites between the parent strains (NCBI accession numbers KU873007 to KU873014) were compared using the muscle pairwise alignment algorithm within the Geneious 9.0.3 platform (47).

Statistical analysis. The number of recombinants, total CFU, and recombination frequency data were analyzed in PROC GLIMMIX (SAS 9.3), which fits statistical models to data with nonnormal distribution and nonconstant variability. For the analysis of frequency, the response distribution was used as the binomial distribution of number of recombinants/total cells. Least-squares differences of means among the treatments were separated by Tukey's honestly significance difference (HSD) test at the significance level $P \leq 0.05$. For the repeated experiments, time factor was used as a random variable. The fringe widths of bacterial colonies among different media also were compared using PROC GLIMMIX in SAS.

Accession number(s). Sequences of the flanking regions of antibiotic cassette insertion sites between the parent strains were deposited in NCBI under accession numbers KU873007 to KU873014.

RESULTS

Growth media influence natural competence in *X. fastidiosa*. Cell suspensions of the two strains were mixed together on agar plates, and recombination was assessed by the acquisition of antibiotic resistance markers.

The results in the solid agar plates of PW, XFM, and PD3 showed that PD3 is more conducive for recombination than the other media (Fig. 1, see also Table S1 in the supplemental material) followed by XFM. The recombination frequency was 1.9 ±
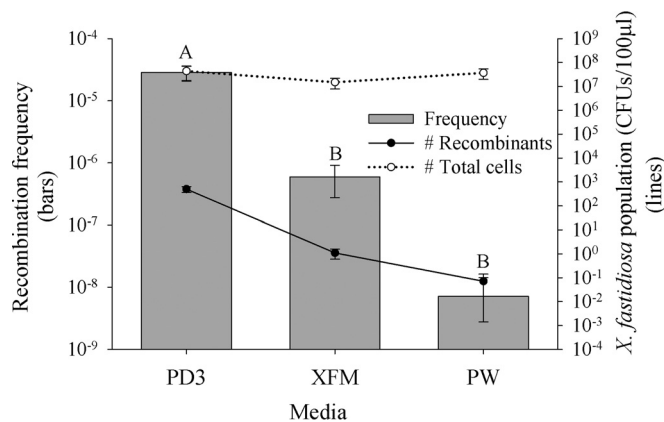
FIG 1 Recombination frequencies, numbers of recombinants, and total CFU counts in different culture media in solid agar plates. Cell suspensions of the two strains were mixed together on agar plates, and recombination was assessed by the acquisition of antibiotic resistance markers. Data represent means and standard errors from different experiments. Experiments were repeated independently twice for XFM ($n = 7$) and at least three times for PD3 and PW ($n = 9$). Different letters above the bars represent significant differences in the recombination frequencies ($P < 0.05$) as analyzed with PROC GLIMMIX in SAS followed by least-squares mean comparisons by Tukey's HSD test.
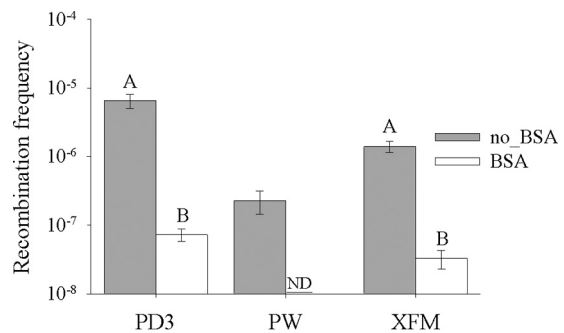

FIG 2 Effect of BSA on recombination. The media used previously (Fig. 1) were modified by addition (PD3) or removal (PW and XFM) of BSA, and a new set of experiments was conducted to measure recombination. Experiments were repeated three times with two to four replications ($n = \geq 8$). Data represent means and standard errors from different experiments. BSA significantly reduced the recombination frequency in each medium. Different letters represent significant differences ($P < 0.05$) within the medium as analyzed with PROC GLIMMIX in SAS. ND, frequency below the detection limit.

0.4×10^{-5} (1.9 recombinants per $\sim 10^5$ cells) for PD3, $2.4 \pm 1.3 \times 10^{-7}$ for XFM, and $8.3 \pm 7.1 \times 10^{-9}$ for PW. Recombinants were readily recovered in PD3 (100 to 3,000 recombinant CFU 100 μl^{-1}). In XFM, the number varied from 0 to 12 recombinant CFU 100 μl^{-1}. In PW, only one recombinant was recovered in all of the experiments performed, and, hence, the recombination frequency of 8.3×10^{-9} was set as the detection limit of the method. The total CFU counts among the three media were not statistically different ($P = 0.22$).

BSA impairs natural competence and twitching motility. Components that are only present in PD3 (sodium citrate dehydrate, succinic acid, and starch) and PW (BSA and L-glutamine) were initially screened for their influence in natural competence as PD3 produced significantly higher number of recombinants than PW. Among the components tested, only BSA had a clear effect, as recombinants could not be recovered from treatments in PW and PW minus L-glutamine, both of which contain BSA but could be recovered from both media when BSA was removed (see Table S1 in the supplemental material). A further test of the effect of BSA was performed in four media (PD3, PD3 plus L-glutamine, PW, and XFM) with and without BSA by conducting additional experiments. Results showed that BSA significantly reduced the frequency of recombination when added to PD3 ($P < 0.001$) (Fig. 2) and PD3 plus L-glutamine ($P < 0.001$) (see Table S1 in the supplemental material) and increased the frequency when removed from PW ($P = 0.0025$) and XFM ($P < 0.0001$) (Fig. 2; see also Table S1). Recombinants were readily recovered in PD3 (323 ± 72 100 μl^{-1}) and in PD3 plus L-glutamine (252 ± 30 100 μl^{-1}) but were significantly reduced when supplemented with BSA (4 ± 0.8 and 4 ± 1.0 100 μl^{-1}, respectively). Similarly, the number of recombinants increased in PW from 0 to 8.7 ± 2.6 100 μl^{-1} and in XFM from 2.0 ± 0.8 to 8.6 ± 2.1 100 μl^{-1} on removal of BSA (see Table S1). Pectin supplementation to PD3 had no significant effect in the recombination frequency ($P = 0.79$) (see Table S1).

Twitching motility, as measured by colony fringe width, was higher in PD3 than in other media (Fig. 3). BSA significantly reduced twitching motility when supplemented to PD3 and significantly increased twitching motility when removed from both PW and XFM with the NS1-CmR mutant (Fig. 3). However, no fringe could be detected with the *pglA*-KmR mutant in any of the media tested (Fig. 3).

Growth environment mimicking *X. fastidiosa* natural habitats supports natural competence. Surface attachment and biofilm formation of the cells were tested in the MCs with PD3 and XFM; PW was not tested as it was not conducive for competence. A mixture of the NS1-CmR and *pglA*-KmR mutants was inoculated into the channels at the beginning of the experiment. Both attachment and biofilm formation were pronounced with PD3, while XFM allowed very poor attachment of cells and formation of biofilms (Fig. 4). Hence, PD3 was selected as the medium to perform further natural competence experiments in MCs.

Within hours of inoculation, abundant *X. fastidiosa* cells were seen attached in the channels with PD3 medium (Fig. 4). Channels were filled with biofilm growth after ~ 5 days of injecting the cells. The frequency of recombination in the MC_in fraction that consists mainly of the biofilm formed under the flow conditions inside the microfluidic channels was equivalent to that under the solid agar condition ($P = 0.52$) (Fig. 5). Noteworthy is the fact that the frequency in the MC_in fraction was significantly higher than in the tubes ($P < 0.0001$) or in the MC_out fraction (consisting of cells collected downstream from the channels) ($P < 0.0001$) (Fig. 5). Recombinants were only occasionally detected in the MC_out fraction, and the frequency was the lowest under this condition.

Natural competence was maintained inside the microfluidic chambers with amendments of grapevine sap. Cell growth in pure xylem sap was slower than in PD3 and in 50% sap; nevertheless, abundant cell aggregation was seen in the MCs after ~ 7 days. However, growth of only the *pglA*-KmR mutant was observed in most of the experiments when pure sap was used, and generally no growth of the NS1-CmR mutant was observed, as revealed by plating of the suspension on separate antibiotic plates at the end of the experiment (data not shown). During one experiment, both mutants were recovered, and recombinants were formed in the MC_in fraction with pure sap ($\sim 16 \pm 2$ recombinant CFU in 100 μl of the culture from six separate plates and a frequency $\sim 3.6 \times$
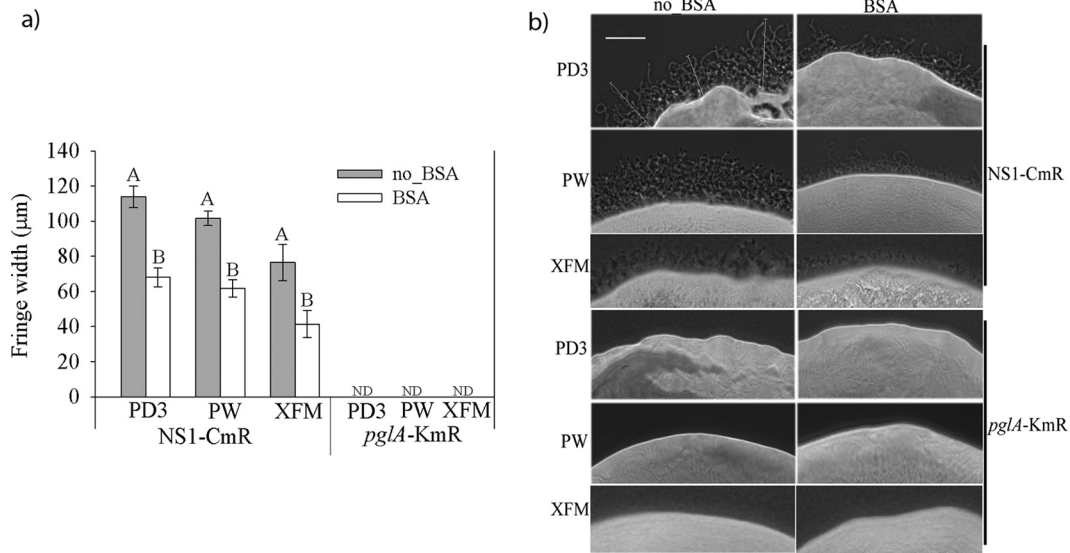
FIG 3 Twitching motilities in media with and without BSA. (a) Fringe width was measured after spotting of the bacteria in media plates and incubating at 28°C for 4 to 5 days for PD3 and PW and 10 to 12 days for XFM. Data represent means and standard errors from different experiments. Experiments were repeated independently three times for PD3 and PW ($n = 29$ to 55) and once for XFM ($n = 4$ to 9). Data were analyzed with PROC GLIMMIX in SAS. Different letters for each bar group indicate significant differences ($P < 0.05$). BSA significantly reduced twitching motility in all three media for the NS1-CmR mutant, while twitching motility was not detectable (ND) for the *pglA*-KmR mutant in any of the media. (b) Colonies of the NS1-CmR (fringe can be seen) and *pglA*-KmR (no fringe) mutants in media with and without BSA. Bar, 100 μm.

10^{-7}) (see Table S1 in the supplemental material). Because of the inconsistent growth of the NS1-CmR mutant in pure sap, 50% sap (diluted with PD3) was used for further experiments. The frequency of recombination was highest in PD3 compared to that in 50% Blanc Du Bois ($P = 0.001$) and 50% Chardonnay sap ($P < 0.0001$) (Fig. 6). Considering only grapevine sap, the frequency of recombination was higher in 50% Blanc Du Bois than in 50% Chardonnay ($P = 0.0002$) (Fig. 6). Noteworthy is the fact that with the sap experiments, recombinants could be recovered only

from the MC_in fraction but not from the MC_out fraction (see Table S1).

Natural competence occurs with heat-killed donor cells. Heat-killed cells of the *pglA*-KmR mutant were used as donors with live cells of the NS1-CmR mutant as recipients and vice versa in PD3 agar plates. Double-antibiotic-resistant recombinant colonies (with a recombination frequency of $3.6 \pm 1.4\,E{-}07$, $n = 5$) were recovered in both of the experiments when NS1-CmR was
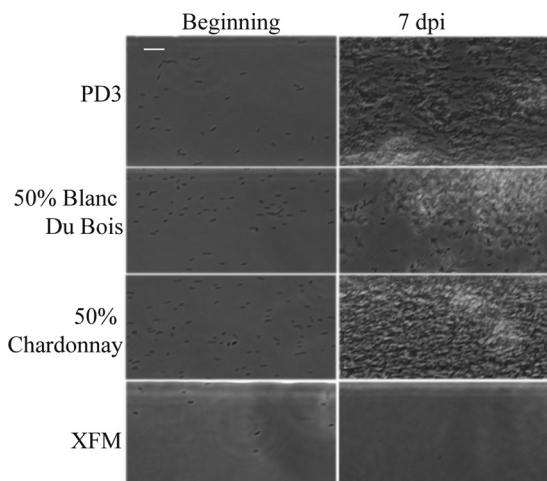

FIG 4 Microfluidic channels showing cell attachment and biofilm formation in media and sap dilutions over time. Mixtures of cells of the NS1-CmR and *pglA*-KmR mutants were injected into the channels. Cells attached well, and copious biofilm growth was observed after 7 days postinoculation (dpi) for both PD3 and 50% sap (susceptible variety, Chardonnay; tolerant variety, Blanc Du Bois) but not for XFM. Bar, 10 μm.
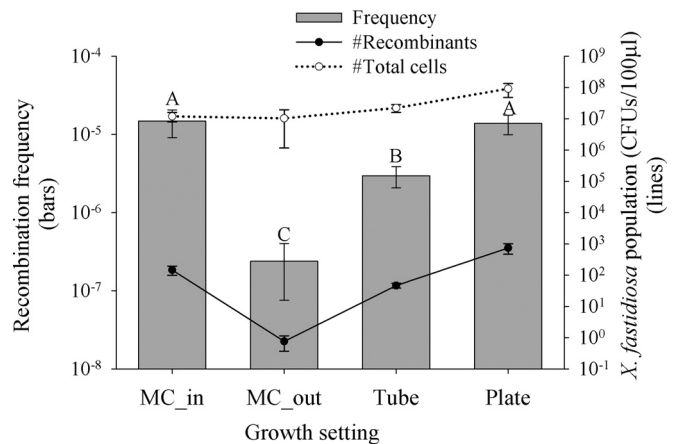

FIG 5 Recombination frequencies, numbers of recombinants, and total CFU in MCs, tubes, and plates with PD3. Mixtures of the two strains were cocultured under the three growth conditions, and recombination at the antibiotic-resistant marker region was assessed. For each growth condition, at least three independent experiments are included with $n = 7, 7, 9$, and 12 for MC_in, MC_out, tube, and plate experiments, respectively. Data represent means and standard errors from different experiments. Different letters indicate significant differences for recombination frequencies as analyzed by PROC GLIMMIX in SAS followed by Tukey's HSD test ($P < 0.05$).
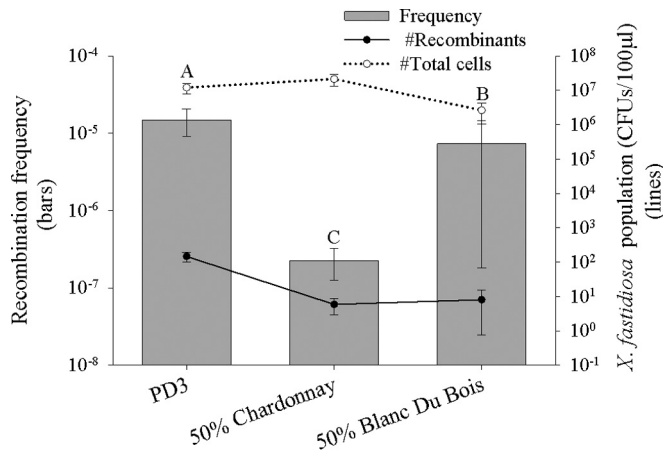
FIG 6 Recombination frequencies, numbers of recombinants, and total CFU from the MC_in fraction with PD3 and 50% grapevine sap. The recombination frequency was highest in PD3 ($n = 7$), followed by 50% sap from tolerant Blanc Du Bois ($n = 2$) and 50% sap from susceptible Chardonnay ($n = 3$), as analyzed in SAS with PROC GLIMMIX and Tukey's HSD test ($P < 0.05$). Data represent means and standard errors for different experiments. PD3 data are included from MC_in data presented in Fig. 5 for comparison.

used as the live recipient, confirming that natural competence is the process facilitating the homologous recombination. No recombinants, however, were obtained when the *pglA*-KmR mutant was used as the live recipient. Experiments were also performed with pAX1.Cm plasmids as the donor DNA and cells of *pglA*-KmR as recipients to confirm the noncompetency of *pglA*-KmR, and no recombinants were detected ($n = 4$, data not shown). Colony PCR of the randomly selected recombinant colonies confirmed that the antibiotic resistance marker gene is inserted in the targeted region by double recombination events (see Fig. S2 in the supplemental material). Comparison of the flanking regions of the antibiotic resistance insertion sites between the parent strains (*pglA*-KmR and NS1-CmR) at the NS1 and *pglA* sites had 99.9% (one mismatch) and 100% identity, respectively (see Fig. S3 in the supplemental material), ruling out the possibility that the noncompetency of *pglA*-KmR is associated with the flanking homology of the recombining region.

DISCUSSION

Several hypotheses have been proposed to explain the existence of natural competence in bacteria. One explanation is that starvation signals induce competence, and the incoming DNA serves as a nutrient source under poor nutrient conditions as demonstrated in *H. influenzae* (6), *Pseudomonas stutzeri* (4), and *R. solanacearum* (5). Based on the results with a minimal medium (XFM) and a rich undefined medium (PW), a previous study (15) speculated that growth in a low-nutrient medium favors natural competence in *X. fastidiosa*. However, the results of this study with these two media (XFM and PW) and PD3, another undefined rich medium, demonstrated that growth in PD3 significantly increases the recombination frequency. This suggests that starvation is not necessary to induce competence in *X. fastidiosa*.

Further investigations of the differences between PD3 and PW were performed by either removing or adding these components to/from one another. Initial screening with the components showed a pronounced effect of BSA on the number of recombi-

nants recovered. Additional experiments confirmed that BSA significantly reduces the recombination frequency when present in PD3, PW, and XFM. Since both XFM and PW contain BSA, this may explain the lower recombination frequencies in these media. In a previous study, BSA had been found to reduce the surface attachment and twitching motility of *X. fastidiosa* (45). In fact, natural competence and twitching motility are dependent on the activity of type IV pili in *X. fastidiosa* (33, 36). Therefore, in this study the correlation between twitching movement and natural competence in different media was investigated. Interestingly, PD3 allowed the highest fringe width, and the presence of BSA significantly reduced twitching motility in all three media. Twitching motility in XFM was lower than in either PD3 or PW as poor growth in XFM resulted in smaller colony sizes. Still the fringe widths of colonies in XFM without BSA were bigger than in XFM with BSA. Most of the colonies spotted in XFM and XFM-BSA showed very little or no visible growth. This can be expected as XFM is a nutrient-limited minimal medium. Moreover, the *pglA*-KmR mutant that did not show twitching movement was not competent when tested with heat-killed NS1-CmR mutant and plasmid DNA as the donor. These results of concomitant decreases in natural competence and twitching motility in BSA-supplemented media and noncompetency of twitch minus strain suggest that twitching motility is correlated with natural competence in *X. fastidiosa*. Natural competence in other Gram-negative bacteria is mediated by type IV pili-like structures (48). In light of the effect of BSA on twitching, it remains to be determined if BSA only alters movement or biogenesis of type IV pili.

Our results with different growth settings showed that the recombination frequency is significantly higher in the MC_in fraction than in the MC_out fraction. The MC_in environment closely mimics xylem vessels and the insect foregut with respect to continuous liquid flow, adhesion of cells on channel walls in a fashion similar to adhesion of cells on xylem vessels and the insect foregut, and formation of biofilms. This environment is conducive for both biofilm formation and twitching motility as demonstrated in previous studies (37, 39). Moreover, expression levels of some of the type IV pili genes were shown to be increased in the MC_in environment compared to those under the other growth conditions (46), implying that activity of type IV pili is increased in this system, which may explain the higher rates of recombination in the MC_in fraction. The MC_out environment, on the other hand, consists mostly of planktonic cells and some detached biofilm fraction from MC_in, which is washed away with the liquid flow. The differences in recombination frequencies in these two environments suggest that the continuous media flow condition of the xylem vessels and growth in biofilm may increase the chances of recombination. Batch cultures in tubes also allowed recombination but at a lower rate than the continuous flow environment of MC_in and surface-attached condition of solid agar plates. A previous study also showed that growth in solid plates increases recombination compared to the growth in the liquid culture tubes (33). Recombinants in the MC_out fraction were recovered when profuse biofilm growth was observed in the MC_in fraction with many recombinants formed. It is possible that the recombinants recovered in the MC_out fraction are due to detachment and washing away of portions of biofilms from the MC_in fractions, supporting the proposition that biofilm formation induces competence. Biofilm formation and quorum sensing signals have been shown to induce natural competence in other

naturally competent bacteria such as *Vibrio cholerae* (49), *Acinetobacter* sp. (50), and *Streptococcus mutans* (51). Biofilms, in addition to having dense populations of cells, contain elevated amounts of extracellular DNA (52), which can be used for transformation by competent cells. Extracellular DNA has been shown to enhance biofilm formation in *X. fastidiosa* (53). Moreover, Kung et al. (33) also showed that a knockout mutant on a biosynthetic gene for diffusible signaling factor (DSF), a cell-cell communication signal in *X. fastidiosa*, had a reduced rate of recombination, implying that a cell-cell communication signal also may be involved in regulating natural competence in *X. fastidiosa*.

MC experiments with grapevine sap provide a closer resemblance to the natural habitat than MCs with the artificial culture medium. Previously, we have shown that the biofilm structure in grapevine sap is more similar to the natural biofilm than are the aggregates observed in synthetic medium inside MCs (54). The experiments with amendments of sap in the MCs detected natural competence, providing an indication that natural competence occurs in the xylem vessels of host plants and possibly in the insect vectors. Although the results with pure sap experiments were not reproducible due to inconsistent growth of one of the strains used, recombinants were recovered once with pure Chardonnay sap as the medium. Recombinants were readily recovered with the 50% sap in PD3 for both tolerant and susceptible varieties. Maintenance of competence with the addition of xylem sap indicates that sap components support DNA acquisition and transformation. Natural competence occurring in environments resembling natural habitats also have been demonstrated in other naturally competent bacteria such as *P. stutzeri* (4) and *V. cholerae* (55), in which artificial medium resembling natural soil extract and natural growth substrate (chitin), respectively, induced competence. In *R. solanacearum*, another xylem-colonizing plant pathogen, natural competence has been demonstrated *in planta* (14), and the recipient strains were shown to have increased virulence, acquiring DNA regions as long as 40 kb from donor strains.

Findings from competence experiments with grapevine sap and the MCs suggest that when two different strains are established together in the xylem vessels or in the vector foregut, recombination is possible. Noteworthy is the fact that in the experiments reported here, recombination was higher with sap from a tolerant grapevine variety, where infection by *X. fastidiosa* is symptomless. Coinfection by two genetically different isolates together in the same plant has been documented before (56), and there are reports of artificial mixed infection of a vector (57) and of a single vector being able to transmit all four subspecies of *X. fastidiosa* (58). Moreover, it was shown that isolates from two different subspecies can cause disease in a single host (59). Hence, the possibility that two different *X. fastidiosa* strains may encounter one another and exchange DNA, as shown by MLST analyses, exists in nature. Donor DNA may be derived from dead cells or may be secreted by a type IV secretion system, as shown in *N. gonorrhoeae* (60). Moreover, the experiment with heat-killed donor cells suggests that recombination is possible if homologous DNA fragments are present in the environment. Although the majority of recombination events will not be beneficial to the recipient cell, some may have adaptive advantages and increased virulence, among other phenotypes under selective pressure. For example, the relatively recent emergence of citrus variegated chlorosis and coffee leaf scorch (in the 1980s) in South America is proposed to be due to intersubspecific recombination between a

X. fastidiosa subsp. *multiplex* donor and an unidentified native recipient based on MLST (30). In addition, strains that are classified in the newly proposed subspecies, *Xylella fastidiosa* subsp. *morus*, that infects mulberry, have been suggested to be generated by recombination between an *X. fastidiosa* subsp. *fastidiosa* donor and an *X. fastidiosa* subsp. *multiplex* recipient (21). A similar mechanism may have resulted in strains that infect blueberry and blackberry (31).

The recombination events observed in this study are based on horizontal acquisition of antibiotic resistance markers (a single gene), which represent a small fraction of the genome of *X. fastidiosa*. Since the natural competence experiments were performed under conditions without any selective pressure, recombination events should be expected to have occurred at other regions of the genome as well but were not detected due to the experimental approach used here. Under the simplistic assumption that gene exchange occurs randomly throughout the genome and with similar frequencies at all loci, the recombination frequencies reported in this study for one locus ($\sim 10^{-5}$ to 10^{-9} recombinants/total cells) could be as much as $\sim 2.5 \times 10^3$ higher, considering the size of the *X. fastidiosa* genome (~ 2.5 Mb).

In summary, *X. fastidiosa* is naturally competent with a high rate of recombination when cultured under the liquid flow conditions of the MC system, which mimics plant xylem vessels and the insect vector foregut. Natural competence in the MCs was maintained even when the medium was supplemented with grapevine xylem sap, suggesting that the natural habitat of *X. fastidiosa* supports natural competence. Moreover, habitats and media that favored increased biofilm growth and increased twitching motility showed increased rates of recombination. This study advances the characterization of the phenomenon of natural competence in *X. fastidiosa* that needs to be further studied to understand the evolution and adaptation of this important plant pathogen.

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