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Impact of Plasmid pQBR103 Acquisition and Carriage on the Phytosphere Fitness of *Pseudomonas fluorescens* SBW25: Burden and Benefit

ANDREW K. LILLEY AND MARK J. BAILEY*

Molecular Microbial Ecology Laboratory, Institute of Virology and Environmental Microbiology, Natural Environment Research Council, Oxford OX1 3SR, United Kingdom

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The phytosphere population densities of *Pseudomonas fluorescens* SBW25EeZY6KX (*lacZX aph xylE*) carrying pQBR103 (Hg^r Tra^+, 330 kbp) declined significantly relative to plasmid-free populations after seed inoculation. As the sugar beet plants matured, ca. 100 days after planting, simultaneous selections for plasmid-carrying hosts were observed in the phyllospheres and rhizospheres of field-grown plants. The recovery of these populations to densities indistinguishable from the densities of plasmid-free inocula (4×10^5 CFU/g in the rhizosphere) demonstrates that phytosphere-associated plasmids confer a specific fitness advantage to host bacteria.

In vitro studies have demonstrated that, in the absence of positive selection, e.g., selection with antibiotics, plasmid carriage can result in reduced growth rates, increased generation times, and predominance (in competition studies) of plasmid-free variants (4, 7, 9, 11, 13). However, smaller reductions in host fitness in soil (8, 12, 18), in agricultural drainage water (23), in compost (1), and on plant surfaces (5, 20) have been noted. Plasmids have often been found to be more stably maintained in these environments than in in vitro studies. These findings can probably be explained by a combination of factors, including low nutrient concentrations, choice of host strains, and environmental selection for (unidentified) plasmid-encoded traits.

Experimental approach. The experimental approach taken here has been to study the effects of plasmid carriage on the ecology of a marked strain, *Pseudomonas fluorescens* SBW25 EeZY6KX (3), colonizing the phytospheres of sugar beet plants. Marked-strains were inoculated onto seeds prior to sowing, and the impact of plasmid carriage on the colonization of various plant habitats was assessed both in terms of the spatial distribution of organisms and in terms of viable counts.

The plasmid chosen (pQBR103, 330 kbp) for this study was acquired by marked *P. fluorescens* SBW25EeZY6KX when it was released to a field crop of sugar beets (16). This self-conjugal plasmid has been characterized as a member of a genetically distinct group (group I) of mercury resistance plasmids that persist from year to year in the microflora of the sugar beet phytosphere at the field site and that are able to transfer between pseudomonads both in vitro and in situ (14–16).

The experimental strain, *P. fluorescens* SBW25, was isolated from sugar beets grown at the Oxford field site and is one of an abundant group of related fluorescent pseudomonads that proliferated over consecutive seasons on root and leaf tissues (3, 21, 22). This strain was chromosomally marked either at two sites by the insertion of the *aph* (kanamycin resistance), *xylE*, and *lacZY* genes (SBW25EeZY6KX) (3) or at one site with tetracycline resistance genes (SBW25ETc) (19). Starter cultures for all experiments were grown from the same glycerol master stocks, which were stored at −70°C. These stocks included fresh associations of plasmids and hosts in order to minimize variation between plasmid-free and plasmid-bearing variants.

The standard methods and materials used in this study have already been described (15). Briefly, plants were randomly selected, divided into three habitats (rhizoplane, immature leaves, and mature leaves), homogenized, decimally diluted, and spread for enumeration of colonies on suitably amended agar media. All counts were expressed per gram and were corrected to sample dry-weight equivalents (soil at 105°C and plant tissues at 85°C; 72 h). Count data were analyzed by sample date by using analysis of variance, with tests for homogeneity of variances (Bartlet's box F test, Cochran's C test, and Hartley's F_{max} test), tests for normality of residuals (normal probability plots), and calculations of coefficients of variation performed with Unistat version 4.56 (Unistat Ltd., London, United Kingdom) and Systat version 5 (Systat, Inc.). Count data were transformed by $\log_{10}$ (CFU/g + 1) to ensure homogeneity of variances.

Greenhouse experiment. Large plant pots (30-cm diameter) were filled with fresh soil from the field site. The top 10 cm of each pot was filled with a 1:1 mixture of field soil and potting compost (no. 1; John Innes, Norwich, United Kingdom). Pots were placed in plastic trays, watered from below, and left to equilibrate for 5 days before planting. At bimonthly intervals pots were fertilized with an N-P-K fertilizer, 13:13:20 (Hydrofertilisers Ltd.). Twelve seeds were planted per pot, and thinning was used in addition to sampling to avoid pot congestion. Seeds were inoculated to similar densities by soaking them for 10 min in saline-washed suspensions of the three experimental strains in six treatments (Table 1), with three replicates per treatment and one pot per replicate. A total of 18 pots were used. Each of the three sets of replicates was organized randomly (2-by-3 arrangement of pots), and the sets were placed on tables approximately 2 m apart in the greenhouse. Ambient greenhouse temperatures were maintained between 16 and 28°C with air conditioning and sunshades. At sampling, one

* Corresponding author. Mailing address: Natural Environment Research Council, Institute of Virology and Environmental Microbiology, Molecular Microbial Ecology Laboratory, Mansfield Rd., Oxford OX1 3SR, United Kingdom. Phone: 44 (0) 1865 512361. Fax: 44 (0) 1865 559962. E-mail: MBJ@mail.nerc-oxford.ac.uk.

TABLE 1. Details of the *P. fluorescens* strains inoculated in six treatments onto sugar beet seeds sown in the greenhouse experiment

Treatment	Strain(s)[a]	CFU (SE)/seed
1	SBW25EeZY6KX	3.75×10^7 (6.5×10^6)
2	SBW25EeZY6KX(pQBR103)	1.93×10^7 (3.3×10^6)
3	SBW25ETc	2.50×10^7 (4.3×10^6)
4	SBW25EeZY6KX SBW25ETc	1.05×10^7 (1.8×10^6) 7.20×10^6 (1.2×10^6)
5	SBW25EeZY6KX(pQBR103) SBW25ETc	3.10×10^7 (5.4×10^6) 1.88×10^7 (3.3×10^6)
6	None	

[a] The relevant characteristics for the strains are *lacZY* Kan^r *xylE* (SBW25EeZY6KX), *lacZY* Kan^r *xylE* Hg^r [SBW25EeZY6KX(pQBR103)], and Tet^r (SBW25ETc).

plant per pot was taken (three per treatment) and three habitats per plant were sampled as described above. Paired-competition treatments were used to improve the sensitivity of the analysis and to avoid natural variability overwhelming the scale of plasmid effects.

No significant ($P > 0.1$) differences between the two plasmid-free strains (whether inoculated alone or in competition) in the colonization of the phytosphere on mature leaves, immature leaves, or the rhizoplane were observed at any time during the study (Fig. 1).

Within each of the three habitats the population densities of *P. fluorescens* SBW25EeZY6KX(pQBR103) were not significantly ($P > 0.05$) different, whether it was introduced alone or in competition with *P. fluorescens* SBW25ETc. However, the densities of the plasmid-bearing strain were significantly ($P < 0.01$) lower than those of both of the plasmid-free strains in leaf habitats at 26 days and in the rhizoplane habitat at 45 days from sowing (Fig. 1). These differences were maintained until day 148, the end of the experiment. The reduction in population density was most marked in the leaf habitats, where plasmid-bearing strains were no longer detected on mature leaves by day 45 and no longer detected on immature leaves by day 84 after introduction. The decline was less severe (but significant) on the rhizoplane, where plasmid-bearing populations fell to 3.5×10^2 CFU g^{-1} by day 84. Following this decline the rhizoplane density of the plasmid-bearing strain increased (Fig. 1). Ratios in individual replicates increased from 1 plasmid-bearing organism per 2,175, 11,300, and 13,500 plasmid-free organisms on day 84 to 1 plasmid-bearing organism per 6, 15, and 19 plasmid-free organisms on day 148. The appearance of plasmidless segregants was assessed by transferring colonies from the isolation media lacking $HgCl_2$ to fresh plates of the same media with and without $HgCl_2$. Segregants grew on the plate without $HgCl_2$ but not on the plate with $HgCl_2$. In this experiment, 200 to 300 colonies were tested per treatment per habitat. No segregants of SBW25EeZY6KX(pQBR103) were detected in any of the greenhouse-derived samples.

The spatial distribution of inocula in the phytosphere was also assessed by pressing roots and leaves sampled at 33 days onto selective agars. Despite counts of plasmid-bearing populations being markedly depressed relative to counts of plasmid-free populations, no differences in the patterns of distribution were noted, as all strains were found on the upper and lower leaf surfaces aggregated in patches covering less than 40% of the surface. On the root, the strains were similarly aggregated,

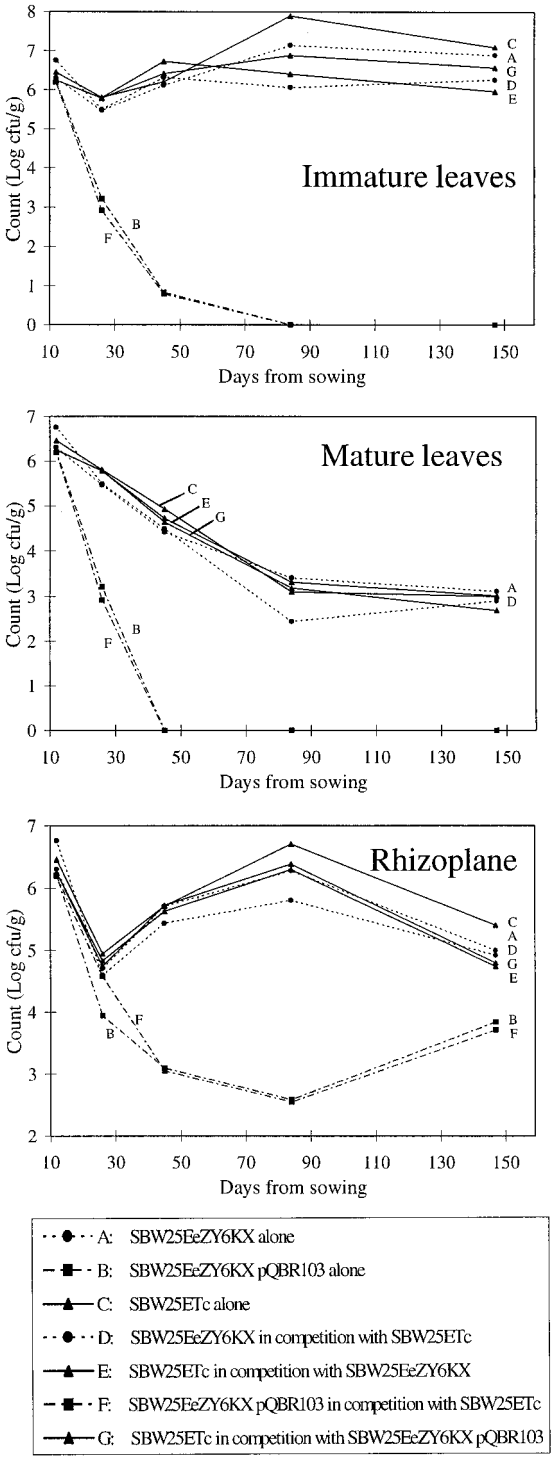

FIG. 1. Colonization of the sugar beet phytosphere by *P. fluorescens* SBW25 strains following inoculation of greenhouse-grown sugar beet seeds. The treatments, inoculated strains, and initial amounts of inocula released are listed in Table 1. Values presented were derived from the means for three sampled plants. Numbers at 12 days are for the entire emerging seedling. Data for the population density of each component strain introduced alone or in competition are presented.

with a particular affinity for zones of secondary root emergence that run vertically down the swollen sugar beet storage root.

Field experiment. As results from greenhouse experiments provide only an indication of behavior in the open environ-

ment, the impact of plasmid carriage on host colonization of the phytosphere in an open field was investigated over an entire growing season. Sugar beets were grown at the Oxford University Farm, Wytham, United Kingdom. The preparation of the soil, seeds, and ground and the applications of fertilizer, herbicide, and pesticide were as described previously (21, 22). Seeds were inoculated and sown to provide nine plots, each containing 100 plants arranged in a randomized Latin square, with one plot in each row and column assigned to each of the three treatments. The seed treatments (with mean numbers of organisms per seed) were (i) inoculation with *P. fluorescens* SBW25EeZY6KX (1.0×10^6 CFU), (ii) inoculation with *P. fluorescens* SBW25EeZY6KX(pQBR103) (1.2×10^6 CFU), and (iii) no inoculation. At sampling, three plants per plot were taken (nine per treatment) and three habitats per plant were sampled as described above.

The colonization and distribution of the inocula in the field-grown plants (Fig. 2) were initially similar to those observed for similar treatments in the greenhouse (Fig. 1). In all three habitats, significantly ($P < 0.05$) lower counts of the plasmid-bearing strain than of *P. fluorescens* SBW25EeZY6KX were observed at 41, 78, and 102 days (Fig. 2). However, for the period from 102 to 148 days, the densities of the plasmid-bearing population on immature-leaf, mature-leaf, and root habitats increased 37,000-, 10,000-, and 2,500-fold, respectively, while plasmid-free-population counts remained steady or declined. By the final sampling time (202 days) the densities of the plasmid-free and plasmid-bearing populations had converged to similar values, where no significant differences were recorded for populations on immature leaves or in the rhizoplane ($P > 0.05$) and a substantially reduced difference was noted for samples taken from mature leaves compared with samples taken at 78 and 102 days. Analysis of variance detected no significant ($P > 0.05$) block effect of the position of plots in the Latin square.

The genetic stability and identity of plasmid pQBR103 were confirmed at the end of the experiment by comparing *Xba*I restriction fragment size patterns of plasmid DNA isolated by the sodium dodecyl sulfate-lysis and sucrose gradient method of Wheatcroft and Williams (24).

The formation of plasmidless segregants was evaluated by taking 200 to 300 colonies from all three habitats per plot per sampling occasion. Plasmidless segregants were detected only on day 167 in mature-leaf samples from two of nine plants (at frequencies of 25 and 70%) and on day 202 in three of nine mature-leaf samples (at frequencies of 3, 14, and 63%) and two of nine immature-leaf samples (at frequencies of 3 and 8%). No plasmidless segregants were detected in any rhizoplane sample.

The spatial distributions of the two inocula were compared by using root and leaf prints at 48 and 181 days. The spatial distributions observed were similar to those on greenhouse-colonized plants. No apparent effect of plasmid carriage could be seen in patterns of colony distribution on leaves or roots.

Summary. The carriage of pQBR103 reduced the plant-colonizing fitness of *P. fluorescens* SBW25EeZY6KX, in both greenhouse and field experiments, on the rhizoplane, the immature-leaf, and the mature-leaf habitats of young sugar beet plants. As plants developed, the observed reduction in relative fitness, as determined by plate count methods, was significantly reversed in all three habitats in the field and on the rhizoplane in the greenhouse. No effect of plasmid carriage on the tissues colonized or on the patterns of colonization could be detected, indicating that the reduction in the viable counts of the plasmid-bearing strain reflected a general decline in fitness which

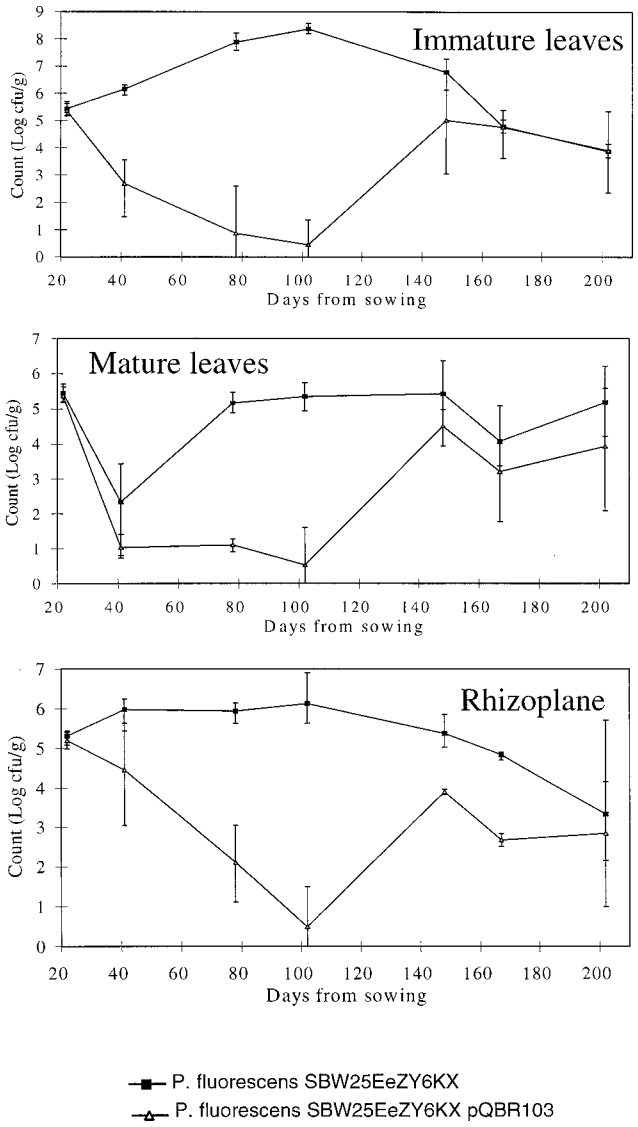

FIG. 2. Colonization of the sugar beet phytosphere by *P. fluorescens* SBW25EeZY6KX and *P. fluorescens* SBW25EeZY6KX(pQBR103) following inoculation of field-grown sugar beet seeds. Values are the means for three replicate plots, each determined by taking and separately assaying the appropriate tissues from three randomly chosen plants ($n = 9$). Maximum and minimum values for the replicate plots are indicated for each point. The counts presented for the mature- and immature-leaf samples at 22 days are the same and derive from counts made of the whole phyllospheres of newly emerged seedlings (cotyledon and stem).

was not phytotissue specific and that the recovery was likewise a general phenomenon.

The basis for the significant recovery of fitness by the plasmid-bearing population on maturing plants has yet to be identified. One possibility is that a burden or net metabolic cost associated with plasmid carriage has been ameliorated by mutations or rearrangements in plasmid and/or host DNA. Examples of increased transconjugant fitness, via these routes, have been reported following prolonged batch or continuous-culture experiments (6, 10, 11, 17). However, the simultaneous recoveries of colonizing fitness by the plasmid-bearing inocula observed in later samples from all plants and in all three habitats make it improbable that the observed successful com-

petition could be driven by a genetic change or mutation occurring spontaneously on individual plants.

The most plausible explanation is that plasmid-encoded traits (as yet unidentified) confer context-dependent benefits that facilitate host survival and colonization in the developing phytosphere. Support for this conclusion can be found in related studies (2, 16) where the isolation of natural transconjugants of *P. fluorescens* SBW25EeZY6KX from the phytosphere was limited to a mid- to late-season period (16). This period of increased conjugative activity and gene flux (2, 16) coincided in both root and leaf habitats with the recovery of the plasmid-carrying population reported here and may indicate a general environmental or habitat-specific selection for beneficial plasmid-conferred traits within a temporal window.

Through greenhouse and field investigations, insight on the impact of a group I plasmid (pQBR103) on the ecology of indigenous pseudomonad populations in phytosphere habitats has been gained. The key observations made demonstrate the capacity of plasmids to generate in permissive hosts novel genotypes that not only modify the behavior of transconjugants but also affect their ability to persist and proliferate. A key task now is the identification and characterization of these plasmid-conferred traits.

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