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Effect of Insertion Site and Metabolic Load on the Environmental Fitness of a Genetically Modified *Pseudomonas fluorescens* Isolate

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An isolate of *Pseudomonas fluorescens* (SBW25) was modified with different marker genes (*lacZY*, *aph-1*, and *xylE*). These marker genes were inserted singly or in combination into two separate (1 Mbp apart) and presumably nonessential sites (-6- and Ee) on the chromosome of SBW25. This allowed the production of a range of genetically modified SBW25 variants that differed with respect to insertion site of the marker genes and metabolic burden. The environmental fitness of the different SBW25 variants was tested in soil, in the rhizosphere of wheat and pea, and on the phylloplane of wheat. Reduced environmental fitness of the different variants was mainly attributed to the extra metabolic burden of novel gene expression, whereas choice of insertion site was of little significance. Changes in environmental fitness were dependent on the environmental conditions; an environment, such as soil, with a low microbial carrying capacity had a negative effect on the environmental fitness of variants with a large metabolic load. In environments with a larger carrying capacity, such as the rhizosphere of pea, environmental fitness of variants with a large metabolic load was not significantly different from that of variants with a smaller metabolic burden.

There is considerable interest in the commercial development of microorganisms for use in bioremediation, as biological control agents, as biofertilizers, or as a means to protect plants against frost damage (13). The ability to create a single organism with a variety of properties from different organisms by using recombinant technology has allowed the possibility of improving existing organisms for the benefit of human society and the environment. However, before such genetically improved organisms can be released into the environment, the wider environmental consequences of such releases need to be determined (21, 22). This is especially important if there is reason to believe that a genetically improved organism is environmentally more fit than the wild-type organism from which it was derived. Environmental fitness describes the interaction of an organism with its environment. The environmental fitness of an organism will differ according to the environment into which it is released. Because naturally occurring permutations in the environment are almost limitless, it is impossible to test environmental fitness empirically in contained environments. As a consequence, the lack of empirical data on the environmental fitness of genetically modified microorganisms (GMMs) has led to legislative measures that are designed to safeguard against the risks associated with their release (22). Such risks include the unforeseen environmental functions of heterologous genes combined with changes in the persistence or impact of inocula.

To overcome the problem of unpredictable behavior of GMMs in the environment, a fundamental understanding of the effects of gene insertions on the GMMs' behavior is required. Experiments using mixtures of GMMs and wild-type organisms often show that GMMs are less fit than the wild-type organisms from which they are derived (6, 7, 19, 24). In some cases, however, no effects (2, 15, 17) and even enhanced survival of a GMM have been reported (12). Several reasons have been suggested for decreased environmental fitness of GMMs. Bromfield and Jones (7) suggested that the decreased environmental fitness of triply marked *Rhizobium* strains was due to a decrease of heterogeneity of the recombinant strain, which would lead to a loss of genetic variability necessary to infect a heterogeneous population of clover. Van Elsas et al. (24) suggested that either expression of the *Bacillus thuringiensis* δ-endotoxin gene (*tox*) in *Pseudomonas fluorescens* was a metabolic burden for the organism or insertion of *tox* into the chromosome led to disruption of essential gene functions, but no evidence for either explanation was given. It generally is assumed that the extra metabolic burden of expressing novel gene sequences is of importance only if expression of the novel gene(s) is maintained at a high level and not directly regulated or if the genes are on plasmids with a high copy number (20, 25). It is thought that under nutrient-deficient conditions, the extra energy demands due to the presence and expression of novel genes in GMMs might lead to large environmental fitness differences. In vitro, however, nutrient stress does not seem to cause differences in environmental fitness between parent and modified strains (8). It also is assumed that any disruption of existing gene functions that are necessary for bacterial growth and survival, due to the introduction of the novel genetic elements into the genome of an organism, can be minimized by appropriate selection procedures during recombinant construction (4).

The aims of the experiments presented in this paper were to clarify factors related to GMM fitness per se and to provide an insight into mechanisms that influence the environmental fitness of GMMs in general. For these purposes we used a range of genetically modified *P. fluorescens* variants that were derived from the same environmental isolate (SBW25). Variants of SBW25 were altered with respect to genetic and metabolic loads by changing the number of gene sequences used and with respect to the positions of the gene sequences on the chromo-

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some (i.e., the same gene sequence was inserted at different positions). By applying mixtures of these variants to contrasting environments which differed in the ability to support the growth of *P. fluorescens* SBW25, the effects of the environments on the fitness of different genetic SBW25 variants could be studied.

MATERIALS AND METHODS

Construction of genetically marked organisms. SBW25, a nonpathogenic, plasmid-free *P. fluorescens* strain, was isolated from the phylloplane of sugar beet (*Beta vulgaris* cv. Amethyst). Two nonessential sites, -6- and Ee, approximately 1 Mbp apart on the physical map of the 6.6-Mbp chromosome of SBW25 (18), were chosen for insertion of the different marker genes by site-directed homologous recombination (2). Four genetic variants derived from SBW25 were constructed for this study: SBW25-6K was made resistant to 0.1% (wt/vol) kanamycin by insertion of the *aph-1* gene into the -6- site of SBW25; SBW25EeK was made resistant to 0.1% (wt/vol) kanamycin by insertion of the *aph-1* gene into the Ee site of SBW25; SBW25-6KX contained at the -6- site of SBW25 the *aph-1* gene for kanamycin resistance coupled to the *xylE* gene, which encodes catechol 2,3-dioxygenase; and SBW25EeZY-6KX contained in addition the *lacZY* genes (expressing lactose permease and β-galactosidase) at the Ee site of SBW25. The Tn*903* kanamycin resistance gene, *aph-1*, was isolated on a 1.4-kbp *Bam*HI fragment from pUC4K (Pharmacia, St. Albans, United Kingdom). The *xylE* gene originated from the TOL(pWWO) plasmid (26) and was modified with the chloramphenicol acetyltransferase promoter (H. Joos, Plant Genetics Systems, Ghent, Belgium). The *lacZY* genes under the control of the *iucA* promoter (5) were isolated from pMON117 (3). All genes were inserted by homologous recombination into the two different sites and were expressed constitutively at high levels (2). Cocultivation in nonselective L broth at 28°C and 1,000-fold dilution daily for 20 days (approximately 200 generations) revealed no significant difference in competitive ability between modified organisms and nonmodified wild-type organisms (2).

Phenotypic characteristics conferred by the inserted genes. The *lacZY* system (1, 10, 11) is one of the most widely used metabolic markers (16). Bacteria of the genus *Pseudomonas* cannot utilize lactose as a carbon source (11), but the insertion of the *Escherichia coli* genes *lacZ* (β-galactosidase) and *lacY* (lactose permease) enables modified organisms to do so. When bacterial colonies expressing the *xylE* gene (catechol 2,3-dioxygenase) are sprayed with a 1% (wt/vol) catechol solution in water, they produce a bright yellow product due to the formation of 2-hydroxymuconic semialdehyde. The kanamycin resistance gene, *aph-1*, provides direct selection of recombinant cells in the presence of 0.1% (wt/vol) kanamycin. When plated onto *Pseudomonas*-selective medium (14) amended with 0.05% (wt/vol) X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 0.1% (wt/vol) kanamycin, the phenotypic characteristics conferred by the different marker genes allowed differentiation of the SBW25 variants. SBW25-6K (*aph-1* at the -6- site) and SBW25EeK (*aph-1* at the Ee site) formed white colonies, SBW25-6KX (*aph-1* and *xylE* at the -6- site) formed white colonies that turned yellow after being sprayed with 1% (wt/vol) catechol, and SBW25EeZY-6KX (*lacZY* at the Ee site and *aph-1* and *xylE* at the -6- site) formed blue colonies.

Culturing and inoculation. SBW25 variants were stored at −70°C on beads treated with cryopreservatives (Technical Service Consultants, Lancs, United Kingdom). Variants were cultured either on tryptic soy agar (Oxoid) for 60 h at 25°C or in tryptic soy broth with shaking (200 rpm) until late log phase. After separation of the bacterial cells from the culture media by repeated washing in 0.25-strength Ringer's solution, cells were suspended in 0.25-strength Ringer's solution to yield a concentration of approximately 10^{10} CFU/ml. Strain combinations were obtained by mixing appropriate cell suspensions together. Wheat seeds (*Triticum aestivum* cv. Axona) or pea seeds (*Pisum sativum* cv. Montana) were soaked overnight in suspensions containing single SBW25 variants or variant mixtures. Before seeds were planted the number of CFU of each strain was estimated by plating a 10-fold dilution range of individually macerated seeds onto agar medium selective for *Pseudomonas* (14) amended with 0.1% (wt/vol) kanamycin and 0.05% (wt/vol) X-Gal (n = 4). Untreated control seeds were soaked in sterile 0.25-strength Ringer's solution. Bacteria were inoculated onto the leaves of spring wheat at tillering, growth stage 22 (23), with a spray application of bacterial cells (approximately 10^9 CFU/ml) suspended in 0.25-strength Ringer's solution and 0.01% (vol/vol) Tween 80.

Environmental fitness of triply marked *P. fluorescens* (SBW25EeZY-6KX) compared with the wild-type organism (SBW25). Seventy-two pots (diameter, 23 cm) were filled with a mixture of 80% silty loam (Hamble series) and 20% grit. Each pot was planted with 10 spring wheat seeds (*T. aestivum* cv. Axona). Plants were grown in a greenhouse at temperatures between 20 and 30°C and a photoperiod of 18 h. Pots were watered daily. At the seedling stage, growth stage 12 (23), plants in 36 pots were sprayed with a suspension of bacteria containing a 50-50 mixture of triply marked recombinant and wild-type SBW25 in 0.25-strength Ringer's solution and 0.01% (vol/vol) Tween 80 (treated). The remaining 36 pots were sprayed with 0.25-strength Ringer's solution and 0.01% (vol/vol) Tween 80 (untreated control). Subsequently, plants from 12 treated and 12 untreated pots were harvested immediately and after 7 and 43 days. Recombinant and wild-type bacteria were quantified by plating a 10-fold dilution range prepared from a weighted, macerated sample of leaves onto a medium selective for *Pseudomonas* (14) amended with 0.05% (wt/vol) X-Gal. After 7 days of incubation at 25°C, the number of CFU of each could be quantified and expressed as a proportion of the total count. Control plants sprayed with 0.25-strength Ringer's solution and 0.01% (vol/vol) Tween 80 were used to determine background populations of indigenous *Pseudomonas* spp.

Effect of site of chromosomal insertion of marker genes on the environmental fitness of recombinant organisms. To investigate if insertion of the novel genes had caused a disruption of essential gene functions, genetic variants containing *aph-1* at either the -6- (SBW25-6K) or the Ee (SBW25EeK) site were compared in the rhizosphere and phylloplane of wheat during competition experiments with the triply marked variant, SBW25EeZY-6KX. Pairwise competition against SBW25EeZY-6KX was necessary because the two kanamycin-resistant variants (SBW25EeK and SBW25-6K) expressed the same phenotype.

Wheat seeds (*T. aestivum* cv. Axona) were soaked in a suspension containing either a 50-50 mixture of SBW25EeZY-6KX and SBW25EeK or a 50-50 mixture of SBW25EeZY-6KX and SBW25-6K. A 10-fold dilution series of five replicate seeds from each treatment was plated onto *Pseudomonas*-selective medium (14) amended with 0.05% (wt/vol) X-Gal and 0.1% (wt/vol) kanamycin. The proportion and inoculum density per macerated seed of each variant were estimated. Into each of 50 10-cm-diameter pots filled with a mixture of 90% silty loam (Hamble series) and 10% grit, three soaked spring wheat seeds (cv. Axona) were planted. Pots were placed in a greenhouse at air temperatures between 20 and 32°C. The photoperiod was 18 h, and plants were watered on a daily basis. Five pots from each treatment were harvested weekly over a period of 35 days, and the population densities of the different genetic SBW25 variants were estimated by plating a 10-fold dilution series of macerated roots onto *Pseudomonas*-selective medium (14) amended with 0.05% (wt/vol) X-Gal and 0.1% (wt/vol) kanamycin.

Effect of metabolic load on the environmental fitness of recombinant organisms. Variants SBW25-6K and SBW25-6KX were mixed together with the triply marked recombinant SBW25EeZY-6KX in 0.25-strength Ringer's solution (SBW25-6K/SBW25-6KX/SBW25EeZY-6KX ratio of 3:4.5:2.5) to create a bacterial suspension containing approximately 10^{10} CFU/ml. Wheat (*T. aestivum* cv. Axona) and pea (*P. sativum* cv. Montana) seeds were allowed to soak overnight in this bacterial suspension (treated) or in sterile 0.25-strength Ringer's solution (control). Soaked individual seeds were planted in small pots (3 cm by 3 cm; 10 cm deep) filled with sieved sandy loam soil (Holiday Hills series). Pots were placed in a growth chamber set at a day temperature of 21°C, a night temperature of 15°C, and a photoperiod of 18 h. Plants were watered daily. Four pots from each treatment were harvested, and a dilution series of a macerated sample of seeds (at 0 days) or roots (at 14 and 28 days) was plated onto *Pseudomonas*-selective medium (14) amended with 0.1% (wt/vol) kanamycin and 0.05% (wt/vol) X-Gal. After 7 days of incubation at 25°C, colonies were sprayed with 1% (wt/vol) catechol in water and the SBW25 variants were quantified according to their distinct phenotypes. Noninoculated control treatments were used to determine the presence of naturally occurring pseudomonads with similar phenotypes.

Comparison of the environmental fitness of recombinant organisms in soil and the rhizosphere. Pea seeds (*P. sativum* cv. Montana) and wheat seeds (*T. aestivum* cv. Axona) were soaked in suspensions with SBW25-6K, SBW25-6KX, and SBW25EeZY-6KX (ratio of 3:4.5:2.5, respectively). Growth conditions and experimental systems were the same as described above. In addition, fallow soil was inoculated with the genetic variants by pipetting 0.1 ml of bacterial suspension onto the soil surface followed by watering. Immediately (at 0 days) and after 14 and 28 days, four pots from each treatment were harvested and a 10-fold dilution series of a macerated sample of seeds (at 0 days) or roots was plated onto *Pseudomonas*-selective medium (14) amended with 0.1% (wt/vol) kanamycin and 0.05% (wt/vol) X-Gal. The different SBW25 variants in samples of seeds and roots were quantified as described above. In addition, a 10-fold dilution series was prepared from each well-mixed sample of fallow soil. After 7 days of incubation at 25°C, colonies were sprayed with 1% (wt/vol) catechol in water and the SBW25 variants were quantified according to their distinct phenotypes. Noninoculated control treatments were used to determine the presence of naturally occurring pseudomonads with similar phenotypes.

The experiments that were carried out to investigate differences in environmental fitness among SBW25 variants are summarized in Table 1.

Statistical analyses. All treatments within each experiment were completely randomized. After normalization of data sets by log or logit transformation [formula for logit transformation, $\ln x/(1-x)$, with x being the proportion of a given strain recovered), data were analyzed by analyses of variance. Where more than two means were compared, significant differences between treatments were analyzed by using least-significant-difference (LSD) values.

RESULTS

Environmental fitness of triply marked *P. fluorescens* (SBW25EeZY-6KX) compared with the wild-type organism (SBW25). Forty-three days after spray application with a 50-50 mixture of the wild type (SBW25) and the triply marked re-

TABLE 1. Experiments carried out to investigate differences in environmental fitness between different SBW25 variants in soil and in the rhizospheres of pea and wheat

Expt	SBW25 strain	Novel gene(s)	Insertion site	Environment
Comparison between wild type and triply marked recombinant	Wild type	None		Wheat phylloplane
	SBW25EeZY-6KX	*lacZY*	Ee	Wheat phylloplane
	SBW25EeZY-6KX	*aph-1 xylE*	-6-	Wheat phylloplane
Effect of insertion site, tested in competition with SBW25EeZY-6KX	SBW25EeK	*aph-1*	Ee	Rhizosphere of wheat
	SBW25-6K	*aph-1*	-6-	Rhizosphere of wheat
Effect of metabolic load	SBW25-6K	*aph-1*	-6-	Rhizospheres of wheat and pea
	SBW25-6KX	*aph-1 xylE*	-6-	Rhizospheres of wheat and pea
	SBW25EeZY-6KX	*aph-1 xylE*	-6-	Rhizospheres of wheat and pea
	SBW25EeZY-6KX	*lacZY*	Ee	Rhizospheres of wheat and pea
Effect of environment	SBW25-6K	*aph-1*	-6-	Rhizospheres of wheat and pea, soil
	SBW25-6KX	*aph-1 xylE*	-6-	Rhizospheres of wheat and pea, soil
	SBW25EeZY-6KX	*aph-1 xylE*	-6-	Rhizospheres of wheat and pea, soil
	SBW25EeZY-6KX	*lacZY*	Ee	Rhizospheres of wheat and pea, soil

combinant (SBW25EeZY-6KX), the wild type became proportionally dominant ($P < 0.001$), accounting for more than 70% of the total introduced *Pseudomonas* population. Therefore, insertion of the gene cassettes *lacZY* and *aph-1–xylE* into the genome of *P. fluorescens* SBW25 resulted in reduced competitive ability of the recombinant strain compared with that of the nonmodified wild-type organism in the phylloplane of wheat.

Effect of site of chromosomal insertion of marker genes on the environmental fitness of recombinant organisms. In competition with the triply marked strain, SBW25EeZY-6KX, no significant differences in environmental fitness between SBW25-6K and SBW25EeK were found in the rhizosphere of wheat, indicating that insertion site choice had little effect on the environmental fitness of the recombinant organisms (Table 2). Both SBW25-6K and SBW25EeK outcompeted the triply marked strain during the 35-day test period ($P < 0.01$). Differences in competitive ability were most pronounced after 21 to 28 days (Table 2).

Effect of metabolic load on the environmental fitness of recombinant organisms. Because no significant interactions between the proportional survival of the SBW25 variants in the rhizosphere of wheat and pea were observed, data obtained for the pea and wheat were subsequently combined and analyzed. Even though proportionally more ($P < 0.01$) SBW25-6KX variants were added to the initial mixture, constitutive expression of the *xylE* gene resulted in populations of this variant proportionally smaller ($P < 0.01$) than those of the SBW25-6K variant after 14 and 28 days (Table 3). Similarly, the triply marked organism, SBW25EeZY-6KX, was less fit ($P < 0.01$) than SBW25-6K. However, no significant difference in environmental fitness was detected in the rhizosphere between SBW25-6KX and the triply marked recombinant SBW25EeZY-6KX after 14 and 28 days, despite the greater metabolic burden of the latter (Table 3).

Effect of environment on the fitness of recombinant organisms. Environmental conditions (soil and rhizosphere of wheat and pea) had a significant effect on the relative survival of the recombinant SBW25 variants SBW25EeZY-6KX, SBW25-6KX, and SBW25-6K. The proportion of the triply marked SBW25EeZY-6KX within the three-membered introduced community declined ($P < 0.001$) rapidly in soil (Table 4). Immediately after inoculation SBW25EeZY-6KX comprised 25% of the total *Pseudomonas* SBW25 community, but this percentage declined to less than 1.6% of the total SBW25 community 28 days after inoculation (Table 4). This did not happen in the rhizosphere of either pea or wheat, where the proportion of the triply marked strain stayed more or less constant (Table 4).

DISCUSSION

The most likely explanation of the decreased environmental fitness of SBW25EeZY-6KX compared with that of the nonmodified wild type was probably the extra metabolic load of expressing the novel phenotypes and possibly the accumulation of novel proteins in the cell. Because marker gene expression is of no direct benefit to GMMs, the extra energy demands imposed on the organisms are not offset by an increased competitive capacity in the environment. The *lacY*, *lacZ*, and *xylE* genes encode lactose permease, β-galactosidase, and catechol 2,3-dioxygenase, respectively, and constitutive expression of these genes might be metabolically expensive. Because kanamycin resistance is conferred by protein production, and thus would put a potential metabolic burden upon the GMM, all subsequent comparisons between the genetically modified SBW25 variants were carried out with organisms that were modified

TABLE 2. Effect of insertion site on environmental fitness of *P. fluorescens* SBW25 variants in the rhizosphere of spring wheat[a]

No. of days after planting	% and logit %[a]	
	SBW25-6K	SBW25EeK
0	40.7 (0.03) a	52.9 (0.12) ab
7	44.9 (−0.33) a	32.3 (−0.82) a
14	59.1 (0.56) ab	70.5 (0.95) b
21	86.2 (1.71) bc	67.8 (0.80) ab
28	76.5 (1.39) bc	86.6 (3.61) c
35	58.3 (0.09) a	60.9 (0.64) ab

[a] SBW25 variants were applied to spring wheat as a seed coating at the time of planting ($n = 5$). Data are expressed as the percentage of SBW25-6K or SBW25EeK variants (logit transformed data in parentheses) in a population containing initially equal proportions of SBW25EeZY-6KX variants (*lacZY xylE aph-1*) and SBW25-6K (*aph-1* at the -6- site) or equal proportions of SBW25EeZY-6KX and SBW25EeK (*aph-1* at the Ee site). Significant ($P < 0.05$) differences between two treatments were obtained by LSD analyses and are indicated by different letters. The overall significance for SBW25-6K was <0.01, and that for SBW25EeK was <0.001.

TABLE 3. Effect of metabolic load on the environmental fitness of *P. fluorescens* SBW25 variants in the rhizosphere[a]

SBW25 variant	% and logit % of total introduced *P. fluorescens* SBW25 population at indicated time (days)		
	0	14	28
SBW25-6K	30.0 (−0.87) a	52.4 (0.13) b	55.3 (0.23) b
SBW25-6KX	44.8 (−0.21) b	22.5 (−1.02) a	20.0 (−1.61) a
SBW25EeZY-6KX	25.3 (−1.10) a	25.1 (−1.26) a	24.8 (−1.14) a

[a] Data were derived from both pea and wheat rhizospheres. SBW25 variants were applied to the seed in similar proportions (SBW25-6K/SBW25-6KX/SBW25EeZY-6KX ratio, 3:4.5:2.5) ($n = 8$). Data are expressed as a percentage of the total introduced *Pseudomonas* population and as the logit transformation of the percentage data (in parentheses). Significant ($P < 0.05$) differences between two treatments were obtained by LSD analyses and are indicated by different letters. The overall significances for values obtained on days 0, 14, and 28 were all <0.01.

with the kanamycin resistance gene (*aph-1*). Addition of the *lacZY* and *xylE* genes, therefore, presented an extra metabolic burden on top of the metabolic burden of expressing *aph-1*. Competition experiments with SBW25-6K (expressing kanamycin resistance) and SBW25-6KX (expressing kanamycin resistance and catechol 2,3-dioxygenase) confirmed that the extra metabolic load of expressing catechol 2,3-dioxygenase caused a decrease in environmental fitness. Surprisingly, no significant difference between the *P. fluorescens* variants SBW25-6KX (expressing kanamycin resistance and catechol 2,3-dioxygenase) and SBW25EeZY-6KX (expressing lactose permease and β-galactosidase in addition to kanamycin resistance and catechol 2,3-dioxygenase) was found in the rhizosphere of pea and wheat.

However, when the organisms were applied to soil, additional expression of the lactose permease and β-galactosidase genes in SBW25EeZY-6KX resulted in a significant reduction in environmental fitness compared with that of SBW25-6KX (Table 4). This suggests that the extra metabolic load of marker gene expression leads to a reduction in environmental fitness only under certain environmental conditions. Previous investigations showed that there was no significant difference in environmental fitness between wild-type SBW25 and the triply marked SBW25EeZY-6KX when the organisms were grown in nutrient broth (9) or in the phytosphere of sugar beet (2). Both these environments might contain enough nutrients for microbial growth to offset the extra metabolic burden of marker gene expression.

Although the relation between metabolic burden and environmental conditions might be difficult to predict, it is clear that an increase in metabolic burden caused by constitutively expressed genes which are of no benefit to the recipient organism is unlikely to lead to an increase in environmental fitness of GMMs. From a risk assessment point of view this is important: increasing the metabolic burden of an organism carries a cost for that organism which might lead to reduced environmental fitness under suboptimal growth conditions. Unless this cost is offset by a clear environmental advantage conferred by the novel genes, the extra metabolic cost will almost certainly result in a reduced performance of the GMM in the environment. Furthermore, taking into account the variability of the natural environment, it is unlikely that an environmental fitness advantage, if this were to occur, would apply to a wide variety of microbial habitats. Extra metabolic activity of expressing novel gene sequences and environmental variability are safeguards against uncontrolled GMM multiplication in the environment. The ability to monitor genetically marked organisms with great sensitivity, however, is of great advantage in ecological and applied studies of microorganisms in the environment. This advantage more than offsets the fact that such genetic modifications might cause a GMM to be slightly less fit than its nonmodified parental strain.

TABLE 4. Effect of different environments on the relative survival of *P. fluorescens* SBW25EeZY-6KX in a mixture of SBW25 variants[a]

No. of days after inoculation	% and logit % of *P. fluorescens* SBW25EeZY-6KX in a mixture of SBW25 variants in:		
	Pea rhizosphere	Wheat rhizosphere	Soil
0	26.7 (−1.11)	23.7 (−1.21)	24.9 (−1.11) a
14	26.9 (−1.01)	14.3 (−2.02)	5.6 (−2.84) b
28	34.7 (−1.11)	28.8 (−0.75)	1.6 (−4.24) c

[a] The mixtures contained SBW25-6K, SBW25-6KX, and SBW25EeZY-6KX in equal proportions. Variant mixtures were applied either to the seed or as a soil drench ($n = 4$). Data are expressed as the percentage of SBW25EeZY-6KX in the total population of SBW25 variants and as logit transformation of the percentage data (in parentheses). Significant ($P < 0.05$) differences between two treatments were obtained by LSD analyses and are indicated by different letters. The overall significance for soil was <0.001; the data for the rhizospheres were not significant.

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