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The structure of a local population of phytopathogenic *Pseudomonas brassicacearum* from agricultural soil indicates development under purifying selection pressure

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Summary

Among the isolates of a bacterial community from a soil sample taken from an agricultural plot in northern Germany, a population consisting of 119 strains was obtained that was identified by 16S rDNA sequencing and genomic fingerprinting as belonging to the recently described species *Pseudomonas brassicacearum.* Analysis of the population structure by allozyme electrophoresis (11 loci) and random amplified polymorphic DNA–polymerase chain reaction (RAPD–PCR; four primers) showed higher resolution with the latter method. Both methods indicated the presence of three lineages, one of which dominated strongly. Stochastic tests derived from the neutral theory of evolution (including Slatkin's exact test, Watterson's homozygosity test and the Tajima test) indicated that the population had developed under strong purifying selection pressure. The presence of strains clearly divergent from the majority of the population can be explained by *in situ* evolution or by influx of strains as a result of migration or both. Phytopathogenicity of a *P. brassicacearum* strain determined with tomato plants reached the level obtained with the type strain of the known pathogen *Pseudomonas corrugata*. The results show that a selective sweep was identified in a local population. Previously, a local selective sweep had not been seen in several populations of different bacterial species from a variety of environmental habitats.

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Introduction

Microorganisms in the environment such as soil are present as populations within communities and interact with the biotic and abiotic compounds of their habitat. A population is characterized by its specific genetic structure and diversity, which in turn is influenced by random genetic drift, selective pressure (i.e. the environmental conditions under which the population has developed) and migration processes. Although the characterization of single isolates from an environmental site would provide information on which species is present in the habitat and what its contribution to the ecosystem could be, it would not allow the effects of the ecosystem on the bacteria and thus their evolution to be determined. For this, the analysis of local populations can provide valuable information. Local populations of several free-living bacteria have been described either with an emphasis on assessing the effect of horizontal gene transfer influencing allelic linkage (Istock *et al*., 1992; Souza *et al*., 1992; Maynard Smith *et al*., 1993; Wise *et al*., 1995; 1996) or with respect to structures of genetic diversity (Paffetti *et al*., 1996; Di Cello *et al*., 1997; Dalmastri *et al*., 1999). Few other studies include analyses with respect to development under selective pressure and migration (Whittam *et al*., 1983; Qiu *et al*., 1997; Wang *et al*., 1999), and only one of these studies (Roberts and Cohan, 1995) deals with free-living bacteria (*Bacillus subtilis* and *Bacillus mojavensis*). This study is based on a worldwide collection of strains (Roberts and Cohan, 1995). In an attempt to characterize the genetic structure with respect to the genetic diversity and evolutionary history (influence of selective pressure and migration) of soil bacterial populations in the closer sense, i.e. the members of a species isolated at a time from one location, we decided to use a rather unspecific isolation protocol for bacteria on a soil sample by taking colonies of the same morphology and colour and applying molecular methods to identify members of the same species. Such a population is then amenable to analysis with methods of different resolutive capabilities [i.e. allozyme electrophoresis, random amplified polymorphic DNA–polymerase chain reaction (RAPD–PCR), metabolic properties and 16S rRNA gene sequence] yielding data for cluster analyses and stochastic tests.

In this communication, we describe the isolation and characterization of a local *Pseudomonas* population from soil. The genetic diversity within this population was as high as the diversity among members of this recently described species isolated in France. We present an analysis of the population structure, indicating that the population developed under a purifying selective pressure that resulted in the high predominance of some haplotypes.

Results

Isolation of strains from soil and initial characterization of the population

A sample of soil (≈250 g) was taken from the A_p horizon of an agricultural field near Kiel (northern Germany). From agar plates (minimal medium with maltose as sole carbon source) incubated with serial dilutions of two independent soil suspensions (separated by 14 days), a total of 177 strains with a highly similar brownish gleaming colony type was isolated [strains 520-1 to 520-42 ($n = 42$) from the first suspension and strains 520-43 to 520-177 ($n = 135$) from the second suspension]. For each of the two isolation procedures, the concentration of total bacterial counts on the initial plates was $\approx 1 \times 10^5$ cells g^{-1} soil. Of these, ≈20% had the brownish gleaming colony type. After further purification and growth on LB medium, 131 of the 177 isolates remained with a highly similar colony morphology and colour. From two strains (520-1 and 520-20), the 16S rRNA gene sequence was determined (≈1470 nucleotides). A database analysis in 1997 showed that the two strains belonged to the genus *Pseudomonas* with *P. corrugata*^{T} being the closest relative (99.43% and 99.64% sequence identity respectively). From an alignment with the 16S rDNA nucleotide sequences of the type strains of 30 *Pseudomonas* species, two primers were designed that allowed part of the 16S rRNA gene specific for *P. corrugata*^{T} and strains 520-1 and 520-20 to be amplified. A subsequent PCR analysis with this specific primer pair showed that all 131 isolates belonged to a population that we initially proposed to be members of the species *P. corrugata*. The 131 purified strains no longer grew on media with maltose as the sole carbon source (see *Experimental procedures*), suggesting that, on the initial isolation plates, other members of the soil bacterial community had provided the conditions for the use of maltose, perhaps by producing extracellular enzymes such as α-amylase, glucoamylase or α-glucosidase.

Allozyme electrophoresis

The 131 environmental strains were examined for their electrophoretic mobility at 11 enzyme loci in non-denaturing gel electrophoresis. A cluster analysis revealed two groups of isolates, A ($n = 119$) and B ($n = 12$) (Fig. 1A). Within group A, three different electrophoretic mobility types were found for both G6P-A and PGM, which separated strain 520-50, strains 520-107 and 520-129 and the other 116 strains (Fig. 1A). Also, for the enzyme PYK-B, three different electrophoretic mobility types were found, which separated strain 520-50, strain 520-129 and the other 117 strains (Fig. 1A). For all other enzymes, the electrophoretic mobility was the same among the 119 strains from group A that were then termed 'main cluster strains'. All strains from group B were identical in their electrophoretic mobility at the 11 tested enzyme loci. A polyphasic taxonomy analysis including DNA–DNA hybridization, 16S rDNA sequence analysis, genomic fingerprinting (RAPD–PCR) and analysis of metabolic properties (API and BIOLOG) showed that the group A isolates belonged to the recently described species *Pseudomonas brassicacearum* (Achouak *et al*., 2000) and that the group B isolates constitute a new species that has been termed *Pseudomonas kilonensis* (Sikorski *et al*., 2001). The inability of the group A isolates to grow on maltose is consistent with the observation that maltose utilization is a variable character in *P. brassicacearum* (Achouak *et al*., 2000).

Genomic fingerprinting by RAPD–PCR

Besides members of our *P. brassicacearum* population, the only well-characterized strains ($n = 10$) of *P. brassicacearum* have been described by Achouak *et al*. (2000). These strains have been isolated from the rhizoplanes of *Brassica napus* and *Arabidopsis thaliana* grown in soils from five different geographical locations in France. The 10 strains could not be differentiated by amplified ribosomal gene restriction analysis (ARDRA) of the amplified 16S rDNA with 11 restriction enzymes (Achouak *et al*., 2000). After determination of 114 phenotypic characters, the 10 strains were subdivided into two phenons, 1a and 1c, with three and two subphenons respectively (see Fig. 1 in Achouak *et al*., 2000). In order to compare the diversity of our *P. brassicacearum* population with the species diversity of *P. brassicacearum*, one strain of each of the five subphenons (coming from at least three different geographical locations) was included as representative strains in a genomic fingerprinting analysis (RAPD–PCR) of 51 strains of the *P. brassicacearum* population (520-50, 520-107, 520-129 and 48 randomly chosen strains). RAPD–PCR was performed with four primers and yielded a total of 71 band positions with all 56 *P. brassicacearum* strains and 60 band positions for the 51 members of our population (Table 1). Most of the latter (47 out of 51) cluster within a

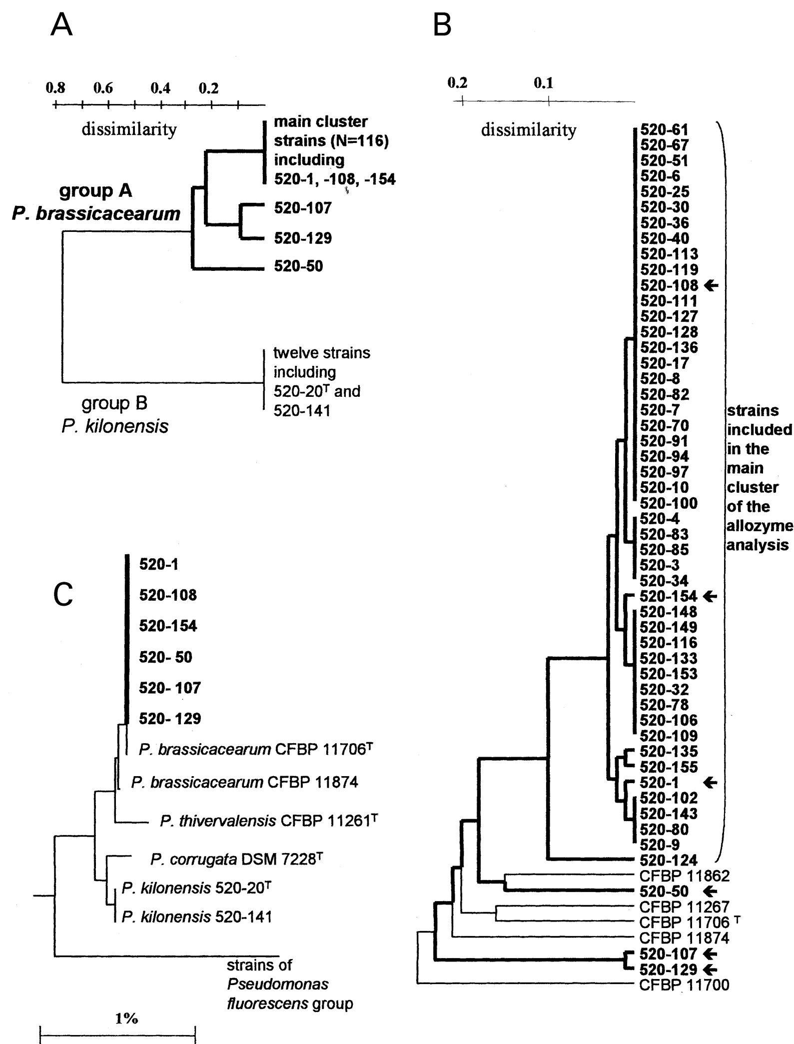

Fig. 1. Genetic relationship of the environmental isolates (bold lines and bold letters) including reference strains of *P. brassicacearum*. (A) and (B) are UPGMA dendrograms based on Nei and Li distance matrices.
A. Allozyme analysis based on 11 enzyme loci and including all environmental isolates of *P. brassicacearum* and *P. kilonensis* (total of 131 strains; listed strain numbers indicate that the 16S rDNA sequence of that strain was determined).
B. RAPD–PCR based on four primers (altogether 71 band positions) and including 51 environmental isolates of *P. brassicacearum* (arrows indicate that 16S rDNA sequence was determined) and five reference strains of *P. brassicacearum*.
C. The dendrogram is based on a neighbour-joining analysis of 16S rDNA sequences [only regions of the rDNA sequences available from all included strains were used (1396 nucleotides, ranging from *E. coli* position 50 to 1445)] of 38 strains out of 30 *Pseudomonas* species (of which only four are shown). The tree was rooted with *E. coli* (accession no. J01695).

similarity value of 0.03 (Fig. 1B). These strains belong to the main cluster observed in allozyme analysis ($n = 116$; Fig. 1A). Closely related to this cluster but already clearly distinguishable is strain 520-124 (Fig. 1B), which is also a member of the main cluster in allozyme analysis (Fig. 1A). In RAPD analysis, strains 520-107 and 520-129 are closely related and can clearly be distinguished from strain 520-50 and from the 'main cluster strains' (Fig. 1B), as already seen in the allozyme analysis (Fig. 1A). Most of the molecular diversity among the 51 members of our population detected by RAPD analysis results from strains 520-50, 520-107, 520-129 and 520-124, as 60% of the band positions (36 out of 60; Table 1) are polymorphic, whereas among the 'main cluster strains' (excluding strain 520-124), only 10% are polymorphic (four out of 39; Table 1). Interestingly, strains 520-50, 520-107 and 520-129 cluster in a similarity range from 0.15 to 0.25 with each other and with the five reference strains from various locations in France (Fig. 1B). Additionally, the proportion of polymorphic sites among the three major lineages of our population (Fig. 1A and B; 55%; represented by strains 520-1, 520-50 and 520-107) was as high as that among the five reference strains from France (62%). This shows that representative lineages of our *P. brassicacearum* population display a similar diversity to that observed in the species. With respect to RAPD haplotypes, the 'main cluster strains' (excluding strain 520-124) add a high proportion of haplotypes (eight out of 12; Table 1 and Fig. 1B) to the diversity of the environmental strains. This was not seen in the allozyme analysis, in which the 'main cluster strains' add only one haplotype to the four haplotypes found in the population (Fig. 1A). Thus, diversification among the 'main cluster strains' was only revealed by RAPD–PCR.

The 12 strains of *P. kilonensis* present in the soil community were also included in this RAPD analysis and were found to be identical in their pattern. This pattern was very different from that of the *P. brassicacearum* population (dissimilarity > 0.7), thus supporting the results of the allozyme analysis (Fig. 1A).

Table 1. Molecular diversity indices as estimated from RAPD analysis of the 56 *P. brassicacearum* strains depicted in Fig. 1B.

	Environmental strains plus reference strains	Environmental strains	Strains from the main cluster
Sample size	56	51	47
No. of haplotypes	17	12	8
No. of band positions	71	60	39
No. of polymorphic band positions	56	36	4

16S rRNA gene sequence and phylogenetic position

The nucleotide sequences of the 16S rRNA gene (≈1470 nucleotides) obtained for the *P. brassicacearum* strains 520-1, -108, -154, -50, -107 and -129 were identical but differed slightly from the recently published 16S rDNA sequences from *P. brassicacearum* strains CFBP 11706^T (AF100321) and CFBP 11874 (AF100322). These have C and G nucleotides at *Escherichia coli* positions 861 and 862, respectively (Achouak *et al.*, 2000), which is very unusual for strains of the genus *Pseudomonas* with almost exclusively G and C nucleotides at these positions. For strain CFBP 11706^T, a C and a G nucleotide have also been reported at *E. coli* positions 947 and 948 (Achouak *et al.*, 2000) instead of G and C present in all other 38 sequences of *Pseudomonas* strains used for the alignment in Fig. 1C. We sequenced the corresponding portion of the 16S rDNA gene (≈440–1080; *E. coli* numbering) of strains CFBP 11706^T and CFBP 11874 and found G and C at both positions (*E. coli* 861/862 and 947/948). We believe that the amended sequences are correct because, otherwise, four mismatching basepairs would be present in regions of the 16S rRNA forming double strands in the putative secondary structure. These data make both *P. brassicacearum* strains with respect to these regions of the 16S rRNA gene identical with the large majority of the *Pseudomonas* sequences. Accordingly, the six environmental strains of *P. brassicacearum* and CFBP 11706^T were identical in their 16S rDNA sequence (Fig. 1C) and differ from CFBP 11874 only at *E. coli* position 1136, where CFBP 11874 has an A instead of a T. The two strains of *P. kilonensis* (520-20^T and 520-141) were identical in their 16S rDNA sequence (Fig. 1C). The sequence data have been deposited in the EMBL database under accession numbers AJ292381, AJ292426, AJ293858 and AJ293859.

Metabolic properties

The metabolic properties (API 20NE, API 50 CH oxidation and API 50 CH fermentation) of eight strains of *P. brassicacearum* (isolates 520-1, -30, -70, -108, -154, -50, -107 and -129) and four strains of *P. kilonensis* (isolates 520-13, -20, -53 and -141; only API 20NE and API 50 CH oxidation) were examined. As observed with allozyme and RAPD analysis, strains 520-50, 520-107 and 520-129 differed from the other 'main cluster strains', which shared the same metabolic properties. Strains 520-50, 520-107 and 520-129 showed only weak oxidase reaction (API 20 NE) in comparison with a strong reaction from the main cluster strains. Additionally, strain 520-50 was the only one able to produce acid from D-raffinose by fermentation (API 50 CH). The strains of *P. kilonensis* showed an identical metabolic profile and differed in several properties from the *P. brassicacearum* strains. Detailed data are published elsewhere (Sikorski *et al.*, 2001).

Phytopathogenic phenotype

The initial taxonomic characterization indicated membership of the 131 environmental isolates to *P. corrugata*. This species is considered a ubiquitous and opportunistic phytopathogenic bacterium with a wide range of host plants. Members are known as the causal agents of

Table 2. Phytopathogenic phenotype of *P. corrugata*^{T} and strain 520-1.

	P. corrugata DSM 7228^T	Strain 520-1	Uninfected control plants
No. of plants in the test	7[a]	7[a]	7
In planta titre (n; $\times 10^7$)[b]	1.9 ± 0.5	3.3 ± 2.3	–
Chlorotic leaflets (%; mean ± SD)	59 ± 6	62 ± 10	15 ± 17
Chlorotic plants	7	6	1
Plants with vascular browning among chlorotic plants	7	4	0
Plants with necrotic lesions among chlorotic plants	5	3	0

a. Each plant was inoculated with 1×10^7 cells.
b. In 5 cm of stem (plant heights about 100 cm).

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tomato and pepper pith necrosis resulting in considerable crop losses (Scarlett *et al*., 1978; Lopez *et al*., 1994). Therefore, *P. corrugata* DSM 7228^T was taken as reference strain in a phytopathogenicity test with tomato plants (Siverio *et al*., 1993; Sutra *et al*., 1997) along with isolate 520-1 (Fig. 1C). At 45 days after infection, the number of colony-forming units (cfu) recovered from a small piece of the plant stem reached or exceeded the infection dose with *P. corrugata* DSM 7228^T or strain 520-1 (Table 2), indicating considerable multiplication in the plant (see *Experimental procedures*), which was at least as high for strain 520-1 as for the tomato pathogen *P. corrugata* DSM 7228^T. The identity of the recovered bacteria with the infecting strains was verified by RAPD–PCR (two primers; three colonies per plant). The few cfu isolated from the uninfected plants did not show any similarity of colony morphology and colour to either *P. corrugata* DSM 7228^T or strain 520-1. Both bacterial strains induced significantly more chlorotic leaflets than occurred in the uninfected control plants ($P = 0.000012$ and 0.00066 respectively; Table 2). Again, the phytopathogenic *P. corrugata* DSM 7228^T was not distinguishable from strain 520-1 in its ability to induce symptoms such as chlorosis ($P = 0.793$), vascular browning and necrotic lesions (Table 2). It was concluded that strain 520-1 of *P. brassicacearum* is phytopathogenic. Considering the very close genetic affinity of the main cluster strains (Fig. 1A and B), it is very likely that all are phytopathogenic. Phytopathogenicity has not yet been reported for *P. brassicacearum*.

The fact that the other 10 published isolates of this species were recovered from the phytoplane of *Brassica napus* or *Arabidopsis thaliana* does not exclude the possibility that the bacteria are phytopathogenic for other plants. As an example, *P. corrugata* is not only phytopathogenic for tomato, but also for pepper and chrysanthemum (Fiori, 1992). Our isolates of *P. brassicacearum* were recovered from soil without apparent association with crucifers. It would be interesting to characterize the phytopathogenicity and host range of other reported isolates of this species (Achouak *et al*., 2000).

Neutrality tests

The neutral mutation–random drift theory of molecular evolution, for brevity called neutral theory, claims that the majority of mutant substitutions that can be observed at the molecular level are the results of random fixation of selectively neutral or nearly neutral mutations, rather than of selective substitutions of definitely advantageous mutations (Kimura, 1968; 1983). A variety of mutation models have been considered, including the infinite-alleles and infinite-sites models. The molecular diversity of our isolates of *P. brassicacearum* was estimated using data from allozyme electrophoresis and RAPD–PCR. For both methods, the haplotypes were defined as alleles and were subjected to tests in order to determine whether the observed allele frequency conformed to the allele frequency expected under neutral development or not (infinite-alleles model). Watterson's homozygosity test with the allozyme data indicated significant deviation ($P = 1.000$; the 95% confidence interval for the expected F is 0.9184; Table 3) of the observed value of homozygosity (F; Table 3, allozyme data) from the value expected under neutral conditions (Table 3), i.e. some haplotypes are represented more frequently than they would be under neutral development. This indicates purifying selection pressure. This was supported by the results from the exact test of Slatkin (1994; 1996), which indicated significant deviations (5% significance level) from the expectation of development under neutrality ($P = 1.000$ and $P = 0.980$ respectively; Table 3) for both allozyme and RAPD data. The homozygosity test did not show significant deviation from neutrality with the RAPD data, although the Watterson's P was rather high ($P = 0.952$; the 95% confidence interval for the expected F is 0.3283; Table 3). Altogether, the test results indicate that the population developed under purifying selection pressure.

Table 3. Ewens–Watterson–Slatkin's tests of selective neutrality on data from RAPD–PCR and allozyme analysis of the environmental isolates of *P. brassicacearum.*

	RAPD analysis	Allozyme analysis
Sample size (*n*)	51[a]	119
No. of haplotypes in sample (*n*)	12	4
Observed *F*-value	0.2902	0.9504
Expected *F*-value	0.1825	0.5790
95% confidence interval for expected *F*	0.3283	0.9184
No. of simulated samples	100 000	100 000
Watterson's homozygosity test	$P = 0.952$[b]	$P = 1.000$[b]
Slatkin's exact test	$P = 0.980$[b]	$P = 1.000$[b]

a. Strains depicted with bold lines in Fig. 1B.
b. Two-tailed test.

Table 4. Tajima's test of selective neutrality on data of RAPD analysis of 51 environmental isolates of *P. brassicacearum.*

Sample size	51[a]
No. of segregating sites (S)/θ_S	36/8.001
Mean no. of pairwise differences (Pi) = θ_π	3.1122
Tajima's D	−2.0580
No. of simulations	10 000
P ($D_{simul} < D_{observation}$)	0.0039[b]

a. Strains depicted with bold lines in Fig. 1B.
b. Two-tailed test.

Tajima's test on selective neutrality based on the infinite-sites model (Tajima, 1989) considers the absence or presence of a band at each band position. Using the RAPD data on 51 members of the *P. brassicacearum* population, the value D of Tajima's test was high and negative ($D = -2.058$; Table 4). The deviation from zero was significant at the 5% significance level [$P = 0.0039$; D lies outside the confidence interval ranging from −1.800 to +2.048; Table 2 in Tajima (1989); Table 4]. The negative D indicates that the population developed under purifying selection pressure (Tajima, 1989) and therefore supports the conclusion drawn from the Slatkin and Watterson analyses.

The fact that the homozygosity test with the RAPD data did not show significant deviation from neutrality results from the particular haplotype configuration of our population and the model the test is based on. The test relies on the haplotype configuration and is therefore very sensitive to single band changes when they occur in one of two otherwise identical RAPD patterns. This occurs frequently within the main cluster of our population. Each of these single band changes will produce a new haplotype and thus a new haplotype configuration. In the Tajima test, the estimator for the population mutation rate based on the number of polymorphic band positions ($\theta_S = 8.001$; Table 4) is very large compared with the estimator based on the mean number of pairwise differences ($\theta_\pi = 3.1122$; Table 4), which leads to an indication of purifying selection (Tajima, 1989). Single changes in band position do not change θ_S and θ_π to an extent that would lead the test to indicate neutrality ($\theta_S \approx \theta_\pi$). The different sensitivities of the tests to single band changes explain the different outcomes of the tests on the same data set.

Discussion

The aim of this study was to analyse the genetic structure of a local bacterial population taken directly from soil in order to determine whether the structure was shaped by selective forces. An initially isolated community of 177 strains from different soil bacteria was identified as consisting of two main populations, one of *P. brassicacearum* (119 members) and one of a new species, *P. kilonensis* (12 members), and other strains not further characterized. The genetic structure of the *P. brassicacearum* population combined with appropriate reference strains was characterized by applying allozyme electrophoresis and RAPD−PCR analysis. Both methods separated the *P. brassicacearum* population into three lineages that were strain 520-50, strains 520-107 and 520-129 and the remaining 'main cluster strains'. The low dissimilarity values from allozyme analysis and RAPD−PCR indicated the close relationship of all members of the population (Fig. 1A and B). This was also supported by the 16S rDNA sequences, which were identical for six representative strains. Clearly, the RAPD analysis provided higher resolution of the population structure than allozyme electrophoresis (compare Fig. 1A and B). We assume that an increase in the number of loci included in the allozyme electrophoresis (which may be difficult to achieve because of the limited number of housekeeping enzymes that can be assayed in native starch gel electrophoresis) would not greatly improve the resolution, whereas the resolution of RAPD−PCR can be increased further by the inclusion of additional RAPD−PCR primers (which are almost unlimited in number). These methodological conclusions based on the analysis of a local population are in agreement with similar findings of the superior resolution capability of RAPD−PCR or restriction fragment length polymorphism (RFLP) analysis compared with allozyme electrophoresis when independent strains of the same species are analysed (Denny *et al*., 1988; Wang *et al*., 1993; Boerlin *et al*., 1995; Hernandez *et al*., 1995).

The neutral theory of evolution claims that the majority of genetic polymorphisms in a population is the result of selectively neutral alleles maintained by a balance between the effects of mutation and random genetic drift (Kimura, 1968; 1983; King and Jukes, 1969; Hartl and Clark, 1997). Based on the neutral theory, several stochastic tests have been proposed to determine whether a population has developed under selectively neutral conditions or whether selection has influenced the genetic structure of a population. Divergence from neutral development of our population was indicated by the homozygosity and Slatkin tests on allozyme data. The RAPD data gave the same result with the Slatkin and Tajima tests, but not with the homozygosity test. The latter was attributed to test characteristics (see *Experimental procedures*) and the specific structure of our population as discussed above (see *Results*). It is concluded that the structure of the population results from development under highly purifying selection pressure, allowing some strains harbouring one (or several) specifically advantageous allele(s) under selection to reach a very high frequency in the population. The power of the selective sweep with which the favourable allele(s) became highly dominant in the population was

apparently so strong that the complete genome was hitchhiked. This allowed the influence of purifying selection during the development of the *P. brassicacearum* population to be detected with methods measuring the overall genetic diversity (allozyme electrophoresis and RAPD−PCR), i.e. covering regions of the genome that are presumably not necessarily in close proximity to the allele(s) under selection. We conclude that the different outcome of the homozygosity test compared with the Tajima test is indicative of the beginning of neutral diversification following purifying selection. The fact that the Slatkin test, relying on the same model as the homozygosity test but applying a different summary statistic, indicated purifying selection supports the notion that neutral diversification among the main cluster strains is just beginning (Fig. 1B).

How can the presence of strains outside the 'main cluster strains' in the population be explained considering the strong purifying selection that has acted upon the population? Perhaps the power or duration of the purifying selection did not suffice to remove all the lineages that made up the original population by *in situ* evolution, and these left-over lineages are represented by strains 520-50, 520-107 and 520-129. Alternatively, if selection was high enough to allow the further existence of only one of the lineages of the original population (main cluster), then strains 520-50, 520-107 and 520-129 could belong to other lineages that have evolved at a different geographical location and are present in our population as a result of migration. Strains of *P. brassicacearum* including the CFBP reference strains analysed here have been isolated from the rhizoplanes of plants grown in the soil in different regions of France (CFBP 11874 and 11862 from Méréville; 11267 and 11700 from Andilly; and 11706^T from Dieulard; Achouak *et al*., 2000). In addition, strains that probably belong to *P. brassicacearum* were recently isolated from soils in Australia (Ross *et al*., 2000). The strains from France were found to be highly similar to some of the environmental isolates from Kiel (Fig. 1B and C). Global migration of soil bacterial strains must be considered a real option (Roberts and Cohan, 1995).

Selective sweeps have been observed among members of worldwide collections of bacteria (Guttman and Dykhuizen, 1994) and eukarya (Nurminsky *et al*., 1998; Rich *et al*., 1998; Depaulis *et al*., 1999). Generally, members of species are subdivided into local populations. For bacteria living in complex habitats, the structures of local populations may differ (Wise *et al*., 1995; Haubold and Rainey, 1996; Helgason *et al*., 1998). Any adaptive mutation will occur first in a local population and will lead within this population to a diversity-purging event, as in the *P. brassicacearum* population studied here. If the advantageous allele(s) is/are not only highly locally, but also in a larger environmental dimension adaptive, then it might lead, after transfer into other local populations by either migration of cells or mechanisms of horizontal gene transfer, to a local sweep within each recipient population (Majewski and Cohan, 1999). Majewski and Cohan (1999) assumed that selective sweeps are rare events and that the duration of the sweep is short relative to the time between sweeps. This may explain why the analysis of genetic diversity of several local populations of bacteria including *Bacillus subtilis*, *Bacillus cereus−B. thuringiensis*, *Burkholderia cepacia* and *Acinetobacter* have so far not provided evidence for strong purifying selection events (Duncan *et al*., 1994; Paffetti *et al*., 1996; Barberio and Fani, 1998; Helgason *et al*., 1998; Dalmastri *et al*., 1999).

Experimental procedures

Isolation of strains

Two separate suspensions of 10% soil were made in sterile 0.9% NaCl (w/v). From serial dilutions of each, aliquots (100 μl) were spread on minimal medium (Lorenz and Wackernagel, 1991) containing maltose as sole carbon source. Plates were incubated anaerobically (AnaeroJar; Unipath) at 28°C for 7 days. Brownish gleaming colonies were chosen and streaked twice on the same medium for single colony isolation at 28°C (anaerobic incubation). Growth of single colonies of the isolates became weaker with each purification step and, after two single colony isolation steps, growth was hardly apparent after 7 days. The residual growth probably resulted from the use of minor organic impurities as carbon source present in the maltose from which the medium was prepared. Cells grown in LB medium (Sambrook *et al*., 1989) were stored in 10% glycerol at −80°C. For the determination of viable counts, serial dilutions of the soil suspensions were plated on LB medium and incubated at 28°C. Cultures of the isolates were grown at 28°C in LB medium on a shaker.

PCR with a primer pair specific for P. corrugata *16S rDNA*

PCR amplification of part of the 16S rRNA gene was carried out in 25 μl volumes containing ≈ 2 ng of genomic DNA, which was prepared with GeneReleaser (Eurogentec) as described by the manufacturer. The reaction mixtures [1 μM each primer AGGTGCTTGCACCTCTTGA (position 78−96, *E. coli* numbering) and primer TCGGTAACGTCAAAACACT AAC (position 470−491, *E. coli* numbering), 50 μM each dNTP (Pharmacia)] contained 0.5 U of *Taq* DNA polymerase (M2868; Promega) in the supplied reaction buffer. The cycling programme with the Robocycler (Stratagene) included an initial heating at 94°C for 5 min and 40 cycles each of 1 min at 94°C, 1 min at 69°C and 2 min at 72°C and a final extension cycle at 72°C for 10 min. PCR products were resolved on agarose gels (1.3%).

Sequencing of the 16S rDNA and phylogenetic analysis

PCR amplification of 16S rRNA genes was carried out in 150 μl volumes containing ≈12 ng of genomic DNA (GeneReleaser; Eurogentec). The reaction mixtures [5% DMSO, 1 μM each universal primers fD1 and rD1 (Weisburg *et al.*, 1991), 50 μM each dNTP (Pharmacia)] contained 1.0 U of *Taq* DNA polymerase (Promega) in the reaction buffer supplied by the manufacturer. The cycling programme in a Perkin-Elmer 480 thermocycler included an initial incubation at 92°C for 5 min, 30 cycles each at 92°C for 2 min, at 58°C for 2 min and at 70°C for 3 min, as well as a final extension at 70°C for 10 min. The PCR product of the environmental isolates was sequenced using primers GTATTACCGCGGCT GCTGGC and GCCAGCAGCCGCGGTAATAC (both positions 517–536 *E. coli* numbering in forward and backward directions) and primer CTCCTACGGGAGGCAGCAG (339–357 *E. coli* numbering). Primer GGGTTGCGCTCGTTACG GG (1096–1114 *E. coli* numbering) was used to sequence part (≈440–1080 *E. coli* numbering) of the PCR product from *P. brassicacearum* strains CFBP 11706^T and CFBP 11874. The DNA sequences were aligned using CLUSTALX 1.8 (Thompson *et al.*, 1997). The alignment was corrected manually. For phylogenetic analysis, regions of the rDNA sequences available for all included strains were used (1396 nucleotides, *E. coli* positions 50–1445). Phylogenetic analyses were performed using a distance-based approach (neighbour-joining method, TREECON software; Van de Peer and De Wachter, 1994).

Allozyme electrophoresis

Cells from overnight shake cultures in 50 ml of LB broth at 37°C were harvested by centrifugation (10 min, 13 000 *g*, 4°C). Pelleted cells were resuspended in 1 ml of ice-cold lysis buffer (10 mM Tris-HCl, pH 6.8, 0.8 mM EDTA, 5 μM NADP) and subjected to sonication on ice (twice for 25 s at 40% duty cycle with 15 s between; Branson sonifier 250; G. Heinemann, Ultraschall- und Labortechnik). Cell debris and unbroken cells were removed by centrifugation in an Eppendorf centrifuge (13 000 r.p.m.) at 4°C for 20 min. The crude lysate was stored at 80°C. Non-denaturing electrophoresis of the crude lysate in starch gels, application of gel running buffers and *in situ* enzyme detection were performed as described earlier (Selander *et al.*, 1986). The following enzymes were used for determination of allozyme variation: shikimate dehydrogenase (SKD), pyruvate kinase (two alleles: PYK-A and PYK-B), glucose-6-phosphate dehydrogenase (two alleles: G6P-A and G6P-B), phosphoglucomutase (PGM), malate dehydrogenase (MDH), malic enzyme (MAE; two alleles: MAE-A and MAE-B), 6-phosphogluconate dehydrogenase (6PG) and adenylate kinase (ADK). For each enzyme, distinctive mobility variants were designated as electromorphs and numbered in order of decreasing rate of anodal migration. Electromorphs were taken as products of alleles at the enzyme loci. Absence of enzyme activity was attributed as null allele (designated as 0). Isolates with distinctive combinations of alleles at the 11 enzyme loci examined corresponded to unique haplotypes. Allozyme patterns were exported into the TREECON software (Van de Peer and De Wachter, 1994) to generate an UPGMA dendrogram from a distance matrix (Nei and Li, 1979).

Genomic fingerprinting by RAPD–PCR

RAPD–PCR was carried out in 25 μl reaction volumes containing buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100 and 0.2 mg ml^{-1} bovine serum albumin), GeneReleaser DNA (1 μl), one of the following primers (0.2 μM), which were (i) CGAGCTTCGCG TACCACCCC; (ii) GTTTCGCTCGATGCGCTACC; (iii) CCC TGGCCGAAATCACCCCC; and (iv) CTACGGCAACGCGAC GCTGAC, *Taq* DNA polymerase (1 U; Promega) and dNTPs (100 μM each; Pharmacia) under a drop of mineral oil (Sigma). An MJ Research PTC-100 cycler (Biozym Diagnostik) was used for amplification: four cycles at 94°C, 40°C and 70°C for 5 min each followed by 30 cycles at 94°C and 55°C for 1 min each and at 70°C for 2 min with a final primer extension at 70°C for 5 min. PCR products were resolved on agarose gels (1.3%). A size marker (Ladder Mix; MBI Fermentas) was used as a reference in all gels. The RAPD patterns were analysed with the software GENE IMAGIR, version 3.52 (Scanalytics), and exported into the TREECON software (Van de Peer and De Wachter, 1994) to generate an UPGMA dendrogram from a distance matrix (Nei and Li, 1979).

Metabolic properties

Enzymatic activities, oxidation and fermentation of carbon substrates and assimilation with carbon substrates were determined using API20 NE and API50 CH strips (bioMérieux Deutschland). Aerobic API strips were read visually after 24 h and 48 h incubation at 28°C; anaerobic strips were read after 148 h at 28°C.

Phytopathogenic phenotype

Tomato (*Lycopersicon esculentum* cv. 'Moneymaker') seeds were germinated in sterilized, moist peaty soil. After 14 days of growth, seven plantlets were infected per bacterial isolate by cutting off the first petiole with a sterile scalpel and applying to the cut surface of the 0.2 mm petiole stump a volume of about 10 μl of a late logarithmic culture in LB medium washed once in a sterile salt buffer (0.85% NaCl in 20 mM phosphate buffer, pH 7.2) and resuspended in the same buffer. The cell number of infection was 1×10^7, which was slightly higher than the 2×10^6 and 4×10^6 cfu in other tests with *P. corrugata* (Siverio *et al.*, 1993; Sutra *et al.*, 1997). In the latter cases, however, the bacteria were injected into the plant stem. The seven control plantlets were treated in the same way, except that sterile buffer was applied instead of the bacterial suspension. During growth, the plants were controlled for the appearance of chlorosis. A plant was recorded as being chlorotic when more than 50% of the remaining leaves exhibited more than 50% chlorotic leaflets each. The symptoms were recorded for up to 45 days after inoculation. For quantifying the *in planta* titre of the infecting bacteria, a 5-cm-long segment of the stem of each infected plant was homogenized with an equal weight of salt buffer by grinding in a mortar to yield a pipettable suspension of ≈1 ml

volume. Serial dilutions of this were plated on LB medium. Colonies were scored after incubation at 28°C for 3 days. The titre obtained that way represents about one-tenth of the total bacteria *in planta* (plant heights of about 100 cm). To verify that the colonies were descendants of the inoculated strain, cells were subjected to PCR identification by comparing their RAPD profiles with the RAPD profile of the strain used for infection. Internal infection symptoms of plants were determined after each plant stem was longitudinally cut with a scalpel and inspected for vascular discoloration and necrotic lesions. Significance tests were performed using unpaired Student's *t*-test.

Statistical analysis and neutrality tests

All statistical analyses including determination of diversity indices and neutrality tests were performed by ARLEQUIN 2.000 (Schneider *et al.*, 2000), a software package for population genetics.

There are two tests based upon Ewens' sampling theory (Ewens, 1972) to determine whether an observed allele frequency configuration conforms to neutral expectation: one is the homozygosity test by Watterson (Watterson, 1978), and the other is an exact test by Slatkin (Slatkin, 1994; 1996). The tests differ in that the homozygosity test incorporates a model of selection to test deviation from the distribution of alleles (allele frequency configuration) expected under the null hypothesis of evolution under neutral conditions (only mutation and random genetic drift, no selection; Watterson, 1977; 1978), whereas the Slatkin test does not. The allele frequency configuration is based on the total number of sampled genes or bacterial isolates (n) and on the observed number of alleles (k). From each allozyme electrophoresis and RAPD–PCR, the haplotypes were defined as alleles. A haplotype is a unique combination of genetic markers present in a chromosome and detectable by a specific experimental procedure. From n and k, the value of homozygosity (F), which is the frequency of pairs of identical isolates among all pairs of isolates in a sample, can be determined. For a data set of $n = 16$ and $k = 7$, possible allele frequency configurations may be '6, 4, 2, 1, 1, 1, 1' or '3, 3, 3, 2, 2, 2, 2' or '10, 1, 1, 1, 1, 1, 1'. In the first example, one allele occurs six times, another four times, a third twice and all other alleles are unique. All possible configurations are generated. Applying Ewens' sampling formula (Ewens, 1972), the probability P_{cj} for each possible configuration to have evolved under neutrality is calculated and compared in two different ways in the homozygosity test and the Slatkin test with the observed configuration. The homozygosity test (Watterson, 1978) uses F as a summary statistic. The probabilities P_{cj} of all configurations having identical or smaller F than the observed configuration are summarized to give the cumulative probability P_H. Assuming an error probability of 5%, the null hypothesis is rejected when $P_H < 0.025$ or $P_H > 0.975$ (a two-tailed test). The exact test (Slatkin, 1994; 1996) is essentially similar to the homozygosity test but, instead of F, it uses the probability of the observed sample ($P_{c\text{-observation}}$) having evolved under neutrality as a summary statistic. The probabilities P_{cj} of all configurations having identical or smaller P_{cj} than $P_{c\text{-observation}}$ are summarized to give the cumulative probability P_E. Rejection of the null hypothesis for the exact test is as for the homozygosity test. The results are in general very close to the homozygosity test. Differences may occur when the P_{cj} of a possible configuration is lower than that of the observed configuration and has an F higher than that of the observed configuration, and vice versa. Therefore, a possible configuration with $P_{cj} < P_{c\text{-observation}}$ is included in the exact test to give P_E but not in the homozygosity test to give P_H, as its F is higher than the observed F. The 95% confidence interval for F was calculated with the program FTEST, which was kindly provided by Dr T. Whittam. The Tajima test (Tajima, 1989) compares two different estimators of the population genetic parameter $\theta = 2N_e\mu$ (where N_e is the effective population size and μ is the mutation rate per sequence per generation), which can be derived independently of the infinite-sites model (θ_S and θ_π). For the application of Tajima's test with RAPD data, both the number of segregating (polymorphic) band positions (S) and the mean number of pairwise differences (Pi) in RAPD sequence (01-string derived from the absence or presence of a band at a respective band position) between the isolates are taken into account to generate θ_S and θ_π respectively. At neutral development of a population, the value D of Tajima's test is zero ($\theta_S \approx \theta_\pi$).

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