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ORIGINAL ARTICLE

Detection and quantification of *Paenibacillus polymyxa* in the rhizosphere of wild barley (*Hordeum spontaneum*) with real-time PCR

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drought tolerance, *Paenibacillus polymyxa*, plant growth-promoting rhizobacteria, real-time PCR, wild barley.

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Abstract

Aim: To detect and quantify the plant drought tolerance enhancing bacterium *Paenibacillus polymyxa* in a collection of 160 *Hordeum spontaneum* rhizosphere samples at the 'Evolution Canyon' ('EC'), Israel.
Methods and Results: PCR primers and a FAM-TAMRA probe (6-carboxyfluorescein, 6-carboxy-tetramethyl-rhodamine) targeting 16S rRNA genes were designed and used to detect and quantify the target strain. Two commercial kits, Bio101 Fast Spin and Mo Bio Ultra Clean Soil DNA, were tested for DNA isolation from the rhizosphere and surrounding soil. Population densities of *P. polymyxa* were studied in the rhizosphere of wild barley and surrounding soil from the contrasting climatic slopes at the 'EC' using the real-time PCR and culture based methods.
Conclusion: *Paenibacillus polymyxa* is one of the best established species in wild barley rhizosphere at the 'EC' slopes. With the real-time PCR assay we are able to detect 1 pg of DNA per PCR corresponding to 100 cells per ml. The results at the 'EC' correlate well to bacterial estimations by culture based methods.
Significance and Impact of the Study: Significantly higher *P. polymyxa* cell number was detected in the rhizosphere of arid 'African' microclimate indicating possible role of adaptive co-evolution with plants.

Introduction

Several *Paenibacillus polymyxa* strains have shown plant growth promoting capability as well various interesting qualities. Hence many research groups have studied *P. polymyxa* strains to exploit their useful functions in agricultural systems, specifically in plant growth promotion and biological control against plant pathogens. *Paenibacillus polymyxa* strains produce numerous secondary metabolites including auxins, cytokinins, lytic enzymes and antimicrobial compounds useful for biotechnological applications (Timmusk and Wagner 1999; Timmusk *et al.* 1999, 2009; Budi *et al.* 2000; Alvarez *et al.* 2006; Haggag and Timmusk 2007; He *et al.* 2007). These bacterial qualities are rather common among plant growth-promoting rhizobacteria. *Paenibacillus polymyxa* B2 is known to enhance plant drought stress tolerance of *Arabidopsis thaliana* and cereals (Timmusk and Wagner 1999 and unpublished data). That rhizobacteria can interfere with osmotic stress regulation has been rarely reported (Timmusk and Wagner 1999; Mayak *et al.* 2004). It holds a huge potential for agricultural applications and we are currently in process to study the complex mechanism of the bacterial ability. It is generally accepted by now that plant roots live in firm teamwork with the surrounding micro-organisms forming a unique self-regulating complex system. Micro-organisms are not only the most abundant organisms in natural systems but are also key players in ecological processes. Hence the underground resources of a plant rhizosphere could provide a basis for

the assessment of issues related to global climate change and species conservation.

In this study, we aimed to develop a robust method for comparison of *P. polymyxa* abundance in wild barley (*Hordeum spontaneum*) rhizosphere grown in normal and drought stressed microclimates. Consequently, the samples were collected from a wild barley rhizosphere at the ecological site 'Evolution Canyon' ('EC'), where drought is the stress factor as well as the evolutionary selection force (http://evolution.haifa.ac.il) (Nevo 1995, 1997, 2001). 'EC' (Fig. 1) is a model presenting sharp ecological contrasts at a microscale, permitting the pursuit of observations and experiments across diverse prokaryote and eukaryote taxa, sharing a sharp microecological subdivision. The 'African' slope (AS) of the 'EC' is north of the equator and receives higher solar radiation than the nearby 'European' north-facing slope (ES) (Pavlicek *et al.* 2003). This solar radiation is associated with higher temperature and drought on the more stressful 'African' slope, causing dramatic physical and biotic interslope divergence, which may have originated several million years ago after mountain uplifts (Pavlicek *et al.* 2003). These canyons are extraordinary natural evolutionary laboratories. Rocks, soils, and topography are similar on the opposite slopes (50–100 m apart at the bottom); the microclimate remains the major interslope divergent factor. Thus 'EC' offers the possibility to study genome evolution and adaptations in contrasting microclimates (Nevo 1995, 1997, 2001; Sikorski and Nevo 2005).

Paenibacillus polymyxa belongs to a group of Gram-positive facultative anaerobic endospore-forming bacteria which taxonomists have separated from the genus *Bacillus* (Ash *et al.* 1993) and transferred to the new genus *Paenibacillus*. These bacteria can form stress-resistant endospores with multilayered cell wall structures; hence, they are able to undergo multiple adaptations that enable them to live under extreme environments. There is significant variation in the abovementioned qualities, allowing the bacteria to inhabit diverse niches within ecosystems (Gardener 2004). Just like several other bacteria, various *P. polymyxa* strains live in the environment as biofilms, which are highly structured, surface-attached communities of cells encased within a self-produced extracellular polymeric substance matrix (Davey and O'Toole 2000; Timmusk *et al.* 2005; Singh *et al.* 2006). Earlier, we characterized the bacterial biofilm produced around barley roots at various time intervals using a model system. The data obtained suggested that the biofilm formation could be involved in the increase of plant tolerance to drought stress by the bacterial strain (Timmusk and Wagner 1999; Timmusk *et al.* 2003, 2005).

Paenibacillus polymyxa has been a bacterium of interest from various viewpoints, and diverse time and labour consuming methods have been employed for its identification and quantification (Lindberg and Granhall 1984; Petersen *et al.* 1995; Bent and Chanway 1998, 2002; da Silva *et al.* 2003). The aim of this study was to quantify the bacteria in numerous climatically contrasting locations at the 'EC'. Hence the reliable and robust TaqMan PCR for quantification in natural systems was required. In the TaqMan assay a probe, is designed to anneal to the target sequence between the classical forward and reverse primers. The probe is dually labelled, with a reporter fluorochrome (6-carboxyfluorescein, or FAM) at one end and a quencher dye (6-carboxy-tetramethyl-rhodamine, or TAMRA) at the 3′ end. In its intact form, the fluorescence emission of the reporter dye will be absorbed by the quencher dye. The probe has a melting temperature approximately 10°C higher than the melting temperature of the primers, in order to anneal to the amplicon during the extension phase of the PCR process. Consequently, the probe will be degraded during the extension phase by the 5′–3′ exonuclease activity of the Taq polymerase. This will result in an increase in reporter fluorescence emission because reporter and quencher are separated. The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR product formation.

Hence the advantage of the real-time PCR method developed here is its high specificity based on primers and probe. PCR product is detected in real time

Figure 1 Cross section of the 'Evolution Canyon' indicating the collection sites on 'African slope' 1 and 2 and 'European slope' 5 and 7.

dependent on fluorochrome degradation and light emission. In employing the method, we quantified bacterial population densities in several *P. polymyxa* spiked soils and at the 'EC' 'African' and 'European' slope microsites.

Materials and methods

Bacterial strains and growth conditions

About 19 *P. polymyxa* strains and 14 representatives of other close and distant genera listed in Table 1 were used for primer specificity studies. Strains were cultured aerobically in nutrient broth or nutrient agar (NA, Difco Laboratories, Detroit, MI, USA). Strains were cultured overnight at 30°C.

Table 1 Bacterial strains used to test the specificity of the real-time PCR assay

Bacterial strain	Origin	C_t value*
Paenibacillus polymyxa B2	Uppsala University, Sweden University of	28·18
P. polymyxa 2b2R	British Columbia, Canada (Dr C.P. Chanway)	28·23
P. polymyxa 18b2R		
P. polymyxa 1, 5, 10, 12, 17, 18, 19, 21, 31, 39, 71, 75, 76, and 79	Research Institute of Bioscience and Biotechnology, Korea (Dr C.M. Ryu)	28·2
P. polymyxa 3239	Agricultural Research Institute, Estonia (Dr H. Laitam)	28·24
P. polymyxa 3224		28·22
P. macerans 3236		39·42
P. durus 3237		38·55
P. peoriae 3238		37·88
P. pumilis PN22	ARC Seibersdorf research GmbH Biore sources, Austria (Dr A. Sessitsch)	40
P. vortex	Tel Aviv University, Israel (Dr Ben-Iacob)	40
P. alvei K 165	Agricultural University of Athens, Greece (Dr S.E. Tjamos)	40
Bacillus cereus UW85	University of Wisconsin, USA (Dr J. Handelsman)	40
Bacillus subtilis		40
P. terrae	Salamanca University (Dr R. Gonzales), Spain	40
P. kribbensis		
P. rhizosphaerius		
P. favisporus		
P. phyllosphaerius		
P. xylanolyticus		

C_t, cycle threshold.
*C_t values when 1 pg of DNA template was used.

Preparation of spores

Spores of *P. polymyxa* strains B2, 2b2R and 3239 were prepared in order to test the DNA extraction method. *Paenibacillus polymyxa* strains were incubated at 30°C for 7 days, on NA supplemented with 10 mg l^{-1} MnSO4. Spores were suspended in Ringer's solution (Merck, Darmstadt, Germany) and pooled. The bacterial suspension was then heat shocked for 20 min at 80°C, cooled, and centrifuged (10 000 ***g*** for 10 min). The presence of spores was controlled microscopically after Shaeffer–Fulton staining (Schaeffer and Fulton 1933). Spore storage was at 4°C.

DNA extraction from cell cultures

Cell culture (2 ml) was harvested in late logarithmic stage and lysed by incubation for 15 min in 150 μl of glucose buffer (50 mmol l^{-1} glucose, 25 mmol l^{-1} Tris-HCl (pH 8), 10 mmol l^{-1} EDTA) with 4 mg ml^{-1} lysozyme. Chromosomal DNA was extracted as described by Ausubel *et al.* (1988) .

DNA extraction from soil samples

The physicochemical properties of the three soils used in this study are presented in Table 2. Fresh soil samples were sieved (2 mm mesh) and stored at 4°C. Nucleic acids were extracted from three 200 mg soil aliquots using two commercial kits, the MoBio (for plants; Carlsbad, CA, USA) and the Fast DNA spin kit (for soil; Bio 101; LaJolla, CA, USA), according to the manufacturers' recommendations. The quality and the size of the soil DNAs were checked by electrophoresis on 1% agarose gels. DNA was quantified using the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE).

Oligonucleotide probe and primer design

An alignment of the 16S rRNA gene sequences from *P. polymyxa* and related species was performed with ClustaIV using megalign 5.01 (DNASTAR, Madison, WI, USA) (Fig. 2). Areas of 100% identity were identified and primers and the TaqMan probe were designed using Primer Express software (Applied Biosystems, CA, USA). The fluorigenic probe is 5′ labelled with FAM (6-carboxyfluorescein) and 3′ labelled with quenching dye -TAMRA (6-carboxyteramethylrhodamine). The primer set amplified a 150 bp region of the 16S rDNA gene. The specificity of primers and probe were checked by using the check probe software provided through the Ribosomal Database project (RDP) and the Basic Local Alignment Search Tool (Blast) network service of GenBank.

Table 2 Soil physico-chemical properties and quantification of *P. polymyxa* in spiked soils. Three different *P. polymyxa* concentrations were spiked into three different soil types. TaqMan PCR was used for C_t number estimations and CFU values were calculated using the standard curve

Soil type	Clay %	Org-C %	Tot-N %	pH	Spiking	Mean C_t*	Standard deviation	CFU g^{-1} soil†
Clay loam	40 ± 1·2	1·4 ± 0·3	0·13 ± 0·23	5·8 ± 0·05	10^2	39·6	0·53	ND
					10^3	29·1	0·64	$10^3 \pm 0{\cdot}4 \times 10^3$
					10^5	19·2	0·71	$10^5 \pm 0{\cdot}5 \times 10^5$
Silty clay loam	39 ± 0·8	4·94 ± 0·4	0·49 ± 0·29	7·8 ± 0·03	10^2	39·9	0·50	ND
					10^3	29·3	0·70	$10^3 \pm 0{\cdot}5 \times 10^3$
					10^5	19·6	0·32	$10^5 \pm 0{\cdot}4 \times 10^5$
Sandy loam	18 ± 0·7	1·9 ± 0·3	0·16 ± 0·024	6·6 ± 0·03	10^2	38·9	0·52	ND
					10^3	29·5	0·42	$10^3 \pm 0{\cdot}4 \times 10^3$
					10^5	19·8	0·51	$10^5 \pm 0{\cdot}4 \times 10^5$
'EC' 'African' slope silty clay	42 ± 1·1	4·68 ± 0·3	1·63 ± 0·29	7·9 ± 0·04		19·62	1·12	$2 \times 10^4 \pm 0{\cdot}5 \times 10^4$
'EC' 'European' slope silty clay	49 ± 0·9	7·49 ± 0·88	4·12 ± 0·33	8·0 ± 0·04		29·6	1·58	$2 \times 10^2 \pm 0{\cdot}6 \times 10^2$

C_t, cycle threshold; ND, not detectable.
*Mean C_t represents three independent DNA isolations and real-time PCR reactions.
†CFUs were calculated using the standard curve shown in Fig 3B.

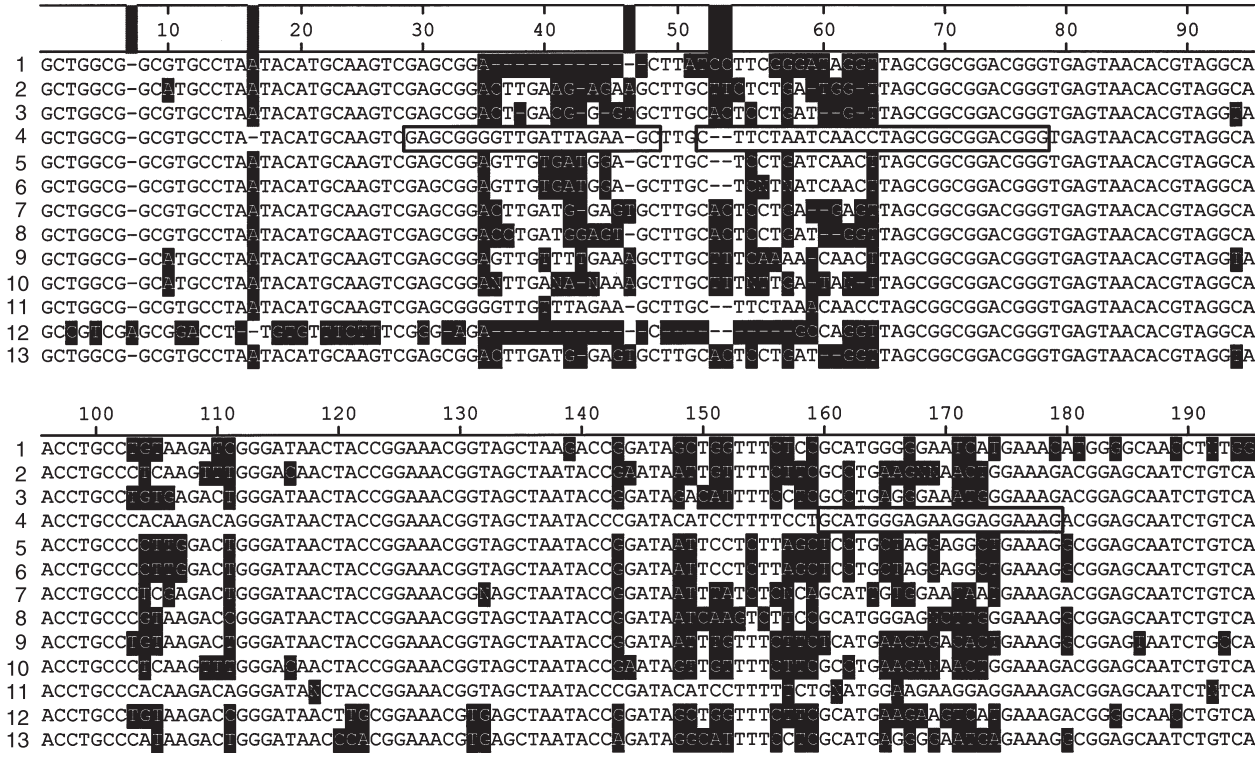

Figure 2 Alignment of the 16S rRNA 190 bp region of various *Paenibacillus* representatives. The 16S rRNA sequences used in the alignment were from (1) *P. validus* S3, DQ836314, (2) *P. amylolyticus* AM237378, (3) *P. apiarus,* (4) *P. polymyxa* B2, (5) *P. durus* AJ251195, (6) *P. jamilae* AJ271157, (7) *P. lautus,* (8) *P. macerans* AM406669, (9) *P. macquariensis* AB073193, (10) *P. pabuli* AM087615, (11) *P. peoriae* AM062691, (12) *P. pulvifaciens* AY530296 and (13) *P. alvei* AY862508. 16S primers (29–48; 159–178) and probe (52–76) used in the study are framed.

Real-time PCR

The PCR amplification was performed using 100 nmol l^{-1} primer 29PpF (5′-GAGCGGGGTTGATTAGAAGC-3′) 150 nmol l^{-1} 179PpR primer (5′-CTTTCCTCCTTCTCCCAT-GC-3′) and 100 nmol l^{-1} 32TM1 FAM/TAMRA probe (5′-CTTCTAATCAACCTAGCGGCGGACGG-3′) (Fig. 2).

The reactions were carried out in 25 μl reaction mixtures using 96-well reaction plates and buffers (Applied Biosystems). The DNA extraction from plant rhizosphere

material was performed in duplicate, and two PCRs were run for each extraction. Dilution series were tested in triplicate.

The 16S rDNA gene fragment amplifications were carried out using five microlitres of diluted template to contain 100 ng of DNA was added to 20 μl of PCR master mix (2·5 μl mixture of 100 nmol l^{-1} forward primer and 150 nmol l^{-1} reverse primer (Invitrogen) and TaqMan probe (100 nmol l^{-1}) (Applied Biosystems) with 5 μl double distilled H_20 added to 12·5 μl of Applied Biosystems TaqMan Universal Master Mix (2× concentrated AmpliTaq Gold DNA polymerase, including uracyl N-glycosylase (UNG), deoxynucleoside triphosphates, passive reference dye, UTP, and optimized buffer). Amplification, data acquisition, and data analysis were carried out in an ABI Prism 7700 Sequence Detector (PE Applied Biosystems). Calf tymus DNA (Sigma) was used as a carrier to test if PCR on high target DNA dilutions was influenced by absorption to tube walls.

PCR initial incubation at 50°C for 2 min to activate the UNG was followed by a 10 min denaturation phase at 95°C. Afterwards, 40-two-step cycles with primer annealing and extension (60°C for 1 min) and denaturation (15 s at 95°C) were performed. The cycle threshold (C_t) values were exported to Microsoft Excel for further statistical analysis.

Real-time PCR sensitivity assay and limit of detection

We used standard curves as the method to determine the detection limit of the assay. About 10 times serial dilutions of *P. polymyxa* B2 logarithmic and stationary growth stages, with and without calf thymus DNA as a carrier were performed. The corresponding CFU per PCR was calculated based on plate counts. For comparison, the dilution series of *P. polymyxa* B2 between 10^7 and 10 were mixed with different concentrations of *B. subtilis* cells to make the final cell density 10^6 CFU ml^{-1}. All determinations were performed in triplicate.

Detection and recovery of *P. polymyxa* spiked into soil

For accuracy of the real-time quantification measurement, three types of soil were studied. *Paenibacillus polymyxa* B2 vegetative cells and *P. polymyxa* B2, 2b2R and 3239 spores were mixed with 5 g of soil. All treatments were established in triplicates. After the treatments, DNA was extracted. The inoculum densities were calculated based on the *P. polymyxa* standard curve and plate counts. DNA from the soil without any spiking with *P. polymyxa* was extracted as a control. The CFU for *P. polymyxa* B2 was determined using standard curve C_t values.

Wild barley *H. spontaneum* rhizosphere sampling and sample preparation

Five wild barley plants were collected from each of the 'AS' and 'ES' locations at 'EC' in April, two weeks before maturation. The plant roots were carefully shaken and washed in sterile distilled water to remove all loosely attached soil and to collect bacteria intimately linked to plant root. Plants were placed in new plastic bags, transferred to the laboratory, and stored at +4°C until processing the next day. 500 mg of the wild barley rhizosphere root samples was then suspended in 1 ml of buffer provided by the manufacturer and homogenized as described by the manufacturer using the FastPrep Instrument (BIO 101). The rhizosphere macerate contains bacteria in wild barley endorhizosphere and very close to the rhizoplane.

Bacterial detection in wild barley rhizosphere samples and bulk soil

Plants were sampled and homogenized as described above. For total *P. polymyxa* numbers the macerates were subjected to DNA isolation and real-time PCR as described above. The serially diluted plant material was Shaeffer–Fulton stained. Spore concentration was checked using a bright line hemacytometer counting chamber (Model 3900, Hausser Scientific Company, Horsham, PA, USA). The culturable aerobic fraction of the total bacterial number in the wild barley rhizosphere was calculated from serially diluted plant material grown at 28°C on ten times diluted tryptic soy agar plates (TSA) with 100 mg l^{-1} cycloheximide for 3 days. For comparison the bacterial number was also detected in bulk soil using the same method.

The real-time PCR as described above was employed to detect *P. polymyxa* in rhizosphere samples. For comparison, *P. polymyxa* identification by culturing was carried out as described by Lindberg and Granhall (1984). Final identification was performed by sequencing the PCR products with universal primers from the 16S rDNA amplification fragment.

Results

Real-time PCR specificity assays

About 14 bacterial stains, some of them phylogenetically closely or distantly related to *P. polymyxa*, were used for primer specificity tests. Two different concentrations of the bacterial pure culture DNA were tested (Table 1). In addition, 19 *P. polymyxa* strains were tested for prime-probe suitability (Table1).

Samples from the closest relatives of *P. polymyxa* (*P. macerans, P. durus,* and *P. peoriae*) had mean C_t

values of 39·42, 38·55, and 37·88, respectively, when 1 pg of template DNA was used (Table 1). When the same amount of *P. polymyxa* DNA was used, the C_t value was 28·2 (Table 1). With smaller quantities of template, the signal was unspecific.

Standard curve and the limit of detection

The *P. polymyxa* primers 29PpF and 179PpR were used to amplify the 16S rDNA 150 bp region (Fig. 2). In order to minimize the C_t values, the concentrations of the primers and of the probe were optimized empirically. The amplifications were optimized to achieve efficiency close to double the product in each cycle. A calibration curve was generated for the bacterial 16S rDNA. The use of a standard curve based on a known concentration of DNA makes it theoretically possible to quantify DNA from any source. In our case, the standard curve was constructed after real-time PCR amplification of six different DNA concentrations ranging from 0·1 pg to 10 ng of DNA per well. C_t values were plotted relative to the corresponding serial dilutions of template DNA extracted from a culture of *P. polymyxa* B2 (Fig. 3a) or to DNA extracted from different cell densities. Target DNA was detectable when the PCR reaction initially contained 1 pg of template. Carrier DNA did not affect the results (data not shown). By using forward and reverse primer concentrations of 100 and 150 nmol l^{-1}, respectively, the C_t values increased linearly with each tenfold dilution of target DNA. Linearity between the C_t values and the DNA concentration was tested over six orders of magnitude dilution series of DNA. This shows that quantification of *P. polymyxa* B2 target DNA was possible over a wide range of concentrations. The slope was −3, 56 and the linear square regression coefficient was 0·999.

Since the environmental samples contain diverse bacterial communities, the quantification of *P. polymyxa* B2 was tested in the presence of non-target *B. subtilis* DNA (Fig. 3b). *Paenibacillus polymyxa* B2 dilutions were mixed with different concentrations of *B. subtilis* cells as described in Materials and Methods. With the assay we were able to detect 1 pg of DNA per PCR corresponding to 100 cells per ml (Fig. 3b). The slope difference was −0·07 (data not shown) for *P. polymyxa* cells versus *P. polymyxa* cells plus non-target *B. subtilis* cells. The low slope difference shows the relatively small amount of error in the presence of non-target DNA.

Detection of *P. polymyxa* spiked into soil samples

Three different soil types (Table 2) were spiked with 10–10^6 *P. polymyxa* B2 cells per gram to test the reliability of the method under natural conditions. Sporulation is a common survival strategy for *P. polymyxa* initiated by complex physiological signals under stressful environmental conditions. Hence the soils were also spiked with *P. polymyxa* B2, 2b2R and strain 3239 spores. These results correlated well with PCR values shown in Fig. 3b (*P. polymyxa* mixed with non-target DNA). The average yield of DNA obtained was 50% if the soils were spiked with vegetative cells (Table 2) and 10% if spiked with spores (data not shown). The two kits did not differ significantly in their DNA isolation efficiency.

Detection of *P. polymyxa* in wild barley rhizosphere samples from the 'EC'

We analysed a collection of 160 rhizosphere samples from 'EC' opposing slope 'AS' and 'ES' microsite 1, 2, 5 and 7 (Nevo 1995, 1997, 2001; Sikorski and Nevo 2005)

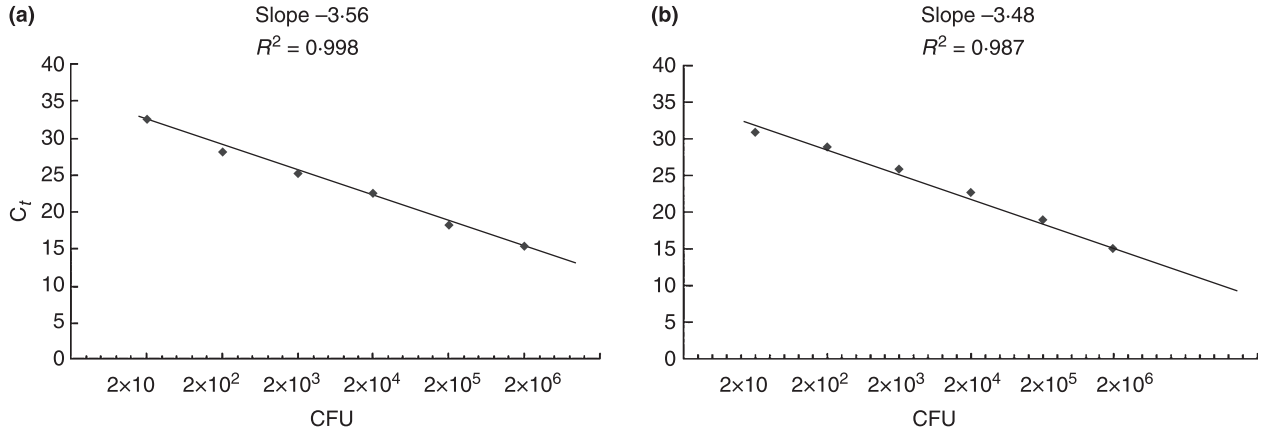

Figure 3 *Paenibacillus polymyxa* standard curve. Serial tenfold dilutions of *P. polymyxa* B2 DNA (a) or cells (b) were analysed in real-time. The C_t values are plotted against the corresponding *P. polymyxa* cell numbers in the PCR. The DNA was extracted from 2×10^6 to CFU per PCR and diluted 10-fold (a). DNA was also extracted from *P. polymyxa* B2 cell serial dilutions and mixed with *B. subtilis* cells to a total of 10^6 CFU (b).

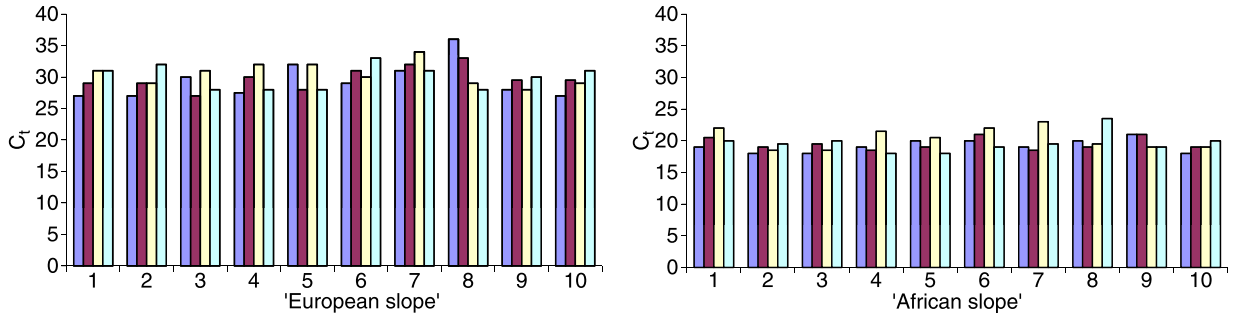

Figure 4 *Paenibacillus polymyxa* density in the rhizosphere of wild barley from the 'African' ('AS') and 'European' slopes ('ES'). C_t values from 10 independent PCR reactions in comparison to the 'AS' microsites 1 and 2 and the 'ES' microsites 5 and 7. Sunny (S) and shady (Sh) locations from both slopes were studied. Note that the more target present in the sample, the smaller the C_t number. The data indicate reproducibly higher *P. polymyxa* density in the rhizosphere of wild barley from the 'AS'. ['European slopes'- (■), 5s; (■), 7s; (□), 5sh; (□), 7sh and 'African slopes'- (■), 2s; (□), 1sh; (□), 2sh].

(Fig. 4). Samples were collected from 10 spots of the well described microsite sunny and shaded locations.

Using a simple standard deviation analysis, we observed relatively small interslope variation in bacterial numbers between microsites as well as between shaded and sunny locations (Fig. 4 and Table 3). In contrast, we detected a dramatic difference in bacterial numbers between the contrasting slopes ('African' and 'European' slope) using the real-time PCR method $2 \times 10^4 \pm 0{\cdot}4 \times 10^4$ bacteria were detected in the rhizosphere of wild barley from the 'African' slope. Remarkably, lower density ($2 \times 10^2 \pm 0{\cdot}5 \times 10^2$) was detected in the rhizosphere samples from the 'European' slope (Fig. 4 and Table 3). By comparison, the 'AS' receives 2- to 8-fold (200–800%) higher radiation than the 'ES' and is drier although the distance between the slopes is 400 m at the top and 100 m at the bottom of the canyon. The *P. polymyxa* presence and quantity is assayed on the 'African' microclimate; microsites 1 (upper portion of the slope) and 2 (mid-slope) and 'European' microsites 5 (lower part of the slope) and 7 (upper part of the slope). On both slopes, samples were collected both on the microsite sunny as well as shady locations (Fig. 4 and Table 3). CFUs were calculated using the standard curve (Fig. 3). Spore percentage was insignificant in wild barley rhizosphere samples (Table 3). Hence the DNA yield from vegetative cells was used for calculations in these samples.

To study if the difference in bacterial numbers could be due to our inability to detect the bacteria at the 'European' slope using the DNA isolation strategy and PCR method, a culture-based method was performed in parallel (see Material and Methods). The bacterial estimates correlated well with the PCR results (Table 3).

Total bacterial number as well the spore number was assayed in 'ES' and 'AS' slopes in sunny and shady areas (Table 3). The assays were performed in rhizosphere samples as well as in bulk soil. We observed that wild barley rhizosphere from both slopes contains less than 1% of spores. At the same time, 'AS' bulk soil contains 55–60% of spores. The corresponding number for 'ES' bulk soil is 7–12%.

Discussion

Real-time PCR

Here we used a method for the rapid bacterial detection in natural environments. The method was successful in detecting *P. polymyxa* cells from laboratory cultures inoculated into environmental samples as well as in detecting and quantifying the presence of the bacterium in the natural laboratory of the opposing slopes of 'EC' (Fig. 1 and Tables 1 and 2). The specificity of the *P. polymyxa* PCR primers and probe was confirmed by homology searches in nucleotide databases and by testing them on 19 *P. polymyxa* strains and on 14 bacterial strains with varying degrees of relatedness to *P. polymyxa* (Table 1 and Fig. 2).

The *P. polymyxa* TaqMan PCR showed high analytical sensitivity, the detection limit being 1 pg in the presence of non-target DNA (Fig. 3). Our detection limit was similar to the other TaqMan applications reported in the literature (Connor *et al.* 2005). The real-time-PCR method we describe offers a detection option that can be completed within 5 h.

Paenibacillus polymyxa is an endospore forming bacterium. Hence, one of the aims of this investigation was to detect spores as well as vegetative cells, and it was important to ensure the efficient release of DNA from the spores. Earlier, this was conducted by microwave treatment, sonication, or germination followed by lysis (Johns *et al.* 1994; Vaid and Bishop 1998). In our study mechanical breaking for 3 min gave the same yield of 10%. The average yield of soil DNA isolation from vege-

Table 3 Bacterial quantification at 'EC' 'African' and 'European' slopes

	'African slope'				'European slope'			
	S		Sh		S		Sh	
	Plant material*	Bulk soil*	Plant material	Bulk soil	Plant material	Bulk soil	Plant material	Bulk soil
P. polymyxa†	$4 \times 10^4 \pm 0{\cdot}4 \times 10^4$	ND	$1{\cdot}5 \times 10^4 \pm 0{\cdot}3 \times 10^4$	ND	$10^2 \pm 0{\cdot}4 \times 10^2$	ND	$1{\cdot}5 \times 10^2 \pm 0{\cdot}4 \times 10^2$	ND
P. polymyxa‡	$2 \times 10^4 \pm 0{\cdot}2 \times 10^4$	$10^2 \pm 0{\cdot}2 \times 10^2$	$9 \times 10^3 \pm 0{\cdot}5 \times 10^3$	$10^2 \pm 0{\cdot}3 \times 10^2$	$10^2 \pm 0{\cdot}3 \times 10$	$10^2 \pm 0{\cdot}3 \times 10^2$	$10^2 \pm 0{\cdot}6 \times 10$	$10^2 \pm 0{\cdot}4 \times 10^2$
Total bacterial count§	$10^6 \pm 0{\cdot}4 \times 10^6$	$10^4 \pm 0{\cdot}2 \times 10^4$	$10^6 \pm 0{\cdot}4 \times 10^6$	$10^4 \pm 0{\cdot}3 \times 10^4$	$10^6 \pm 0{\cdot}3 \times 10^6$	$10^4 \pm 0{\cdot}5 \times 10^4$	$10^7 \pm 0{\cdot}2 \times 10^7$	$10^4 \pm 0{\cdot}2 \times 10^4$
Spore ratio to vegetative cells¶ (%)	0·5	55	0·6	60	0·7	7	0·5	12

ND-not determined; Ct, cycle threshold; S, sunny; sh, shady.
*The data are expressed per gram of fresh weight. Wild barley roots were prepared as described in Materials and Methods.
†Bacterial numbers calculated using the Ct numbers and standard curve shown in Fig 3B Each data point represents 10 independent TaqMan PCR reactions.
‡Bacterial numbers calculated using culture based methods Each data point represents three independent experiments.
§The culturable aerobic fraction of the total becterial CFUs was determined on TSA plates as described in Material and Methods.
¶Spores were counted using Shaeffer–Fulton staining.

tative cells using the MoBio and FastDNA kits was 50%. Hence, the detection limit of added bacteria in practice was about 100 cells (Fig. 3B). At the same time, even lower numbers of added *P. polymyxa* cells produced the amplification product. Thus, the detection level was lower, but the quantification of cells was not possible under 100–200 CFU g^{-1} plant material. DNA extraction from the rhizosphere and soil samples can be one of the major hurdles for real-time PCR. Most of the simple extraction methods result in impure DNA-containing natural fluorescence, e.g. from soil humic substances. The MoBio and FastDNA kits used for DNA extraction from gave an ultra pure DNA. The problem associated with the commercial kits is the relatively low recovery, especially concerning spore extractions. As the spore percentage in wild barley rhizosphere samples is very small, and the bacterial recovery rate calculated was similar, using culture based methods; the kits are very applicable for current study (Table 3). We are currently working to ensure the high purity good recovery DNA extraction method from bulk soil where spore ratio is high.

'Evolution Canyon'

Employing the real-time PCR method, we showed that *P. polymyxa* was present in the rhizosphere of wild barley in reproducibly higher numbers on the dry 'AS' in comparison to the 'ES' (Fig. 4 and Table 3). We also showed that *P. polymyxa* forms quite a large portion from all cultivable bacteria in wild barley rhizosphere: roughly 10^4 at 'AS' and 10^2 at 'ES' (Table 3). Total bacterial count in the rhizosphere macerate varies from 10^6 to 10^7. Furthermore, the rhizosphere macerate Shaeffer–Fulton staining and microscopic visualization revealed that spores form less than 1% of total bacterial counts in the 'AS' as well as in the 'ES' (Table 3). These results are expected since, with the rhizosphere sampling (see Materials and Methods), we aimed to eliminate the disturbance of loosely attached micro-organisms and thus studied the bacteria intimately linked to plant root and root exudates. This outcome is in agreement with our earlier work where we showed that *P. polymyxa* is highly compatible in natural environments due to its well-known antibiotic substance production and endophytic ability (Timmusk *et al.* 2005).

The result that the bacterial number was significantly higher in the rhizosphere of the stressful 'African' microclimate is an interesting finding. Climate has been regarded as one of the most important evolutionary forces driving biodiversity in nature. The earlier observed wild barley enhanced drought stress tolerance at the 'AS' is certainly mediated by a complex network of transcription factors and other regulatory genes that control multiple defense enzymes, proteins, and pathways (Rizhsky *et al.* 2004;

Suzuki *et al.* 2005). What could be the ecological significance of the microclimatic fluctuations shift in *P. polymyxa* root population? It is known that a rhizosphere is an ecosystem exposed to fluctuations due to shifts in composition on root exudates which have marked influence on microbial communities (Lynch 1990; Van Loon 2007). Bulk soil is considered a nutritionally poor environment to which some bacteria may adapt by forming resting or dormant cells or spores. Plants can excrete a strong selection over soil micro-organisms in order to select favoured groups that could optimize their survival (Lynch 1990; Bergsma-Vlami *et al.* 2005). This type of selection has most likely occurred at the 'EC' wild barley rhizosphere since the rhizobacteria on the ancient site have co-evolved with their host over millennia. *Paenibacillus polymyxa* colonizes plant roots as biofilms (Timmusk *et al.* 2005). We speculate that the higher *P. polymyxa* number and biofilm layers formed on the dry 'AS' could serve as an adaptation against desiccation. *Paenibacillus polymyxa* number on the 'ES' rhizosphere macerate didn't differ much from its number in bulk soil. It is known that the major component in biofilms is water (up to 97%) and the characteristics of the solvent are determined by the solutes dissolved in it (Zhang *et al.* 1998; Sutherland 2001). The plant in turn supplies the bacteria with root exudates to ensure that most of the biofilm is kept in an active physiological state. Consequently, the root exudation enables *P. polymyxa* to colonize as large numbers of vegetative cells (Table 3). The spore percentage is relatively small in 'AS' rhizosphere samples, especially compared to the spore ratio in bulk soil (Table 3).

It is currently widely accepted that rhizobacteria in several plant–rhizobacterial combinations induce changes not only in colonized root but the whole plant (Persello-Cartieaux *et al.* 2003). Earlier we observed that *P. polymyxa* B2 inoculation correlates with a striking increase in drought stress responsive ERD15 and RAB18 expression (Timmusk and Wagner 1999). It is possible that, in addition to protective biofilm formation, the higher bacterial number on the stressful 'AS' is the quorum required to induce changes in a complex network of transcription factors and other regulatory genes that control multiple drought-tolerance related pathways. Recent reports suggest that genes controlling stomata closure could be involved in plant osmotic stress tolerance enhancement (Vahisalu *et al.* 2008). How wild barley enhanced drought tolerance on the AS is related to *P. polymyxa* biofilm formation and structure as an adaptive strategy for wild barley requires further critical testing.

In conclusion, our results show that *P. polymyxa* is one of the best established species in wild barley rhizosphere on the 'AS' and 'ES'. Quantification of *P. polymyxa* in environmental samples using methods currently available has been labour-intensive. The TaqMan real-time PCR for *P. polymyxa* rapid detection and reliable quantification is a tool which will enable our understanding of the bacterial function in plant adaptation in natural systems.

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