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Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification

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Abstract

Real-time quantitative PCR assays were developed for the absolute quantification of different groups of bacteria in pure cultures and in environmental samples. 16S rRNA genes were used as markers for eubacteria, and genes for extracellular peptidases were used as markers for potentially proteolytic bacteria. For the designed 16S rDNA TaqMan assay, specificity of the designed primer-probe combination for eubacteria, a high amplification efficiency over a wide range of starting copy numbers and a high reproducibility is demonstrated. Cell concentrations of *Bacillus cereus*, *B. subtilis* and *Pseudomonas fluorescens* in liquid culture were monitored by TaqMan-PCR using the 16S rDNA target sequence of *Escherichia coli* as external standard for quantification. Results agree with plate counts and microscopic counts of DAPI stained cells. The significance of 16S rRNA operon multiplicity to the quantification of bacteria is discussed. Furthermore, three sets of primer pair together with probe previously designed for targeting different classes of bacterial extracellular peptidases were tested for their suitability for TaqMan-PCR based quantification of proteolytic bacteria. Since high degeneracy of the probes did not allow accurate quantification, SybrGreen was used instead of molecular probes to visualize and quantify PCR products during PCR. The correlation between fluorescence and starting copy number was of the same high quality as for the 16S rDNA TaqMan assay for all the three peptidase gene classes. The detected amount of genes for neutral metallopeptidase of *B. cereus*, for subtilisin of *B. subtilis* and for alkaline metallopeptidase of *P. fluorescens* corresponded exactly to the numbers of bacteria investigated by the 16S rDNA targeting assay. The developed assays were applied for the quantification of bacteria in soil samples.

Keywords: TaqMan-PCR; SybrGreen-PCR; Total counts of bacteria; Peptidase genes; 16S rDNA copy number; Soil bacteria

1. Introduction

The quantification of microorganisms and especially those with certain physiological functions in complex biological samples until now has mainly been performed by culture dependent techniques such as the determination of cfu (colony forming unit) or MPN (most probable number) on selective media (Bach and Munch, 2000). These methods are biased by the fact that growth conditions are never suitable for obtaining visible growth of all the present bacterial species or strains. Consequently, results of such

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investigations are strongly dependent on the applied culture conditions. Furthermore, the need for cultivation may be quite undesirable in time-critical applications such as clinical microbiology diagnosis or food quality control in production processes. To overcome these problems, the culture independent polymerase chain reaction (PCR) has been used for the quantification of genes as markers for bacteria, either by the estimation of PCR product yield on agarose gel (Johnsen et al., 1999; Li and Drake, 2001) or by MPN-PCR (Rosado et al., 1996). However, both techniques are laborious and time consuming, and allow only a limited number of samples to be analyzed at the same time. The TaqMan PCR has evolved to a promising tool for quantification, enabling the accurate analysis of 96 samples without the need for gel electrophoresis at the beginning of the (most reliable) exponential phase of amplification. This quantitative PCR technology uses the 5′ exonuclease activity of the *Taq* DNA polymerase to digest an internal fluorogenic probe annealed to the target sequence during primer elongation and allows the calculation of the starting copy number in the sample by comparison with fluorescence in standard samples of known target concentrations.

This technology has been successfully used for the quantification of *varicella zoster* virus (Hawrami and Breuer, 1999; Ryncarz et al., 1999), *Ehrlichia phagocytophila* (Pusterla et al., 1999), or *Salmonella* (Kimura et al., 1999) in pure cultures. In this study, we present the first application of this technique for detecting and quantifying total bacteria by targeting universal bacteria specific 16S rDNA regions and even physiological groups of bacteria with conserved functional genes by using degenerate primers. An oligonucleotide set which is suitable for this application however, has to fulfill certain requirements in terms of base composition, annealing temperatures, length of the generated amplicons and the position of the internal probe in the target regions. Therefore, new 16S rDNA targeting oligonucleotides should be designed.

We have recently published degenerate PCR primers and internal probes for PCR amplification and dot blot detection of bacterial genes for extracellular peptidases, which had also been designed with the prospect of the potential use for TaqMan quantification (Bach et al., 2001). It has been shown that from almost all of the proteolytic bacteria, isolated from three different soils, at least one of the peptidase class genes, namely subtilisins (*sub*) and neutral (*npr*) and alkaline (*apr*) metallopeptidases, could be detected by applying these oligonucleotides. Most of the investigated *Bacillus* spp. had both, *sub* and *npr* genes and in the investigated *Flavobacterium–Cytophaga* strains even all three peptidase classes could be detected. We have also shown that these genes can be specifically amplified from soil DNA.

The aim of this study was to apply the TaqMan-PCR technology to the culture independant quantification of peptidase genes in relation to the amount of 16S rRNA gene copies representing the size of the bacterial population in complex habitates. The development of oligonucleotides for 16S rRNA gene quantification is described in this paper as well as the application of the previously established primer/probe sets for the quantification of peptidase genes.

2. Material and methods

2.1. Organisms and growth conditions

The strains for qualitative DNA extraction listed in Table 1 were cultivated in 6 ml volumes of the appropriate medium and under the recommended culture conditions at 140 rpm over night. The strains used for the validation of the quantitative PCR assays (Table 5) were grown in 300 ml of 1/2 nutrient broth medium (Merck, Darmstadt, Germany) at 30 °C with shaking at 140 rpm. At the beginning of the stationary growth phase, samples were collected for investigation. For the extraction of genomic DNA, cell suspensions were pelleted by centrifugation at $4500 \times g$ for 10 min at 6 °C. The pellets were stored at −20 °C until used for extraction of genomic DNA.

2.2. Conventional bacterial measurements

Cfu were counted as visible colonies obtained by plating 100 μl of serial ten fold dilutions of the cell suspensions on agar plates (1/2 nutrient broth medium, Merck) and after 3 days of incubation at 30 °C. Total cell counts were determined after membrane filtration and staining with 4′,6 diamidino-2-phenylindole

Table 1
PCR mediated generation of amplicons using the 16S rDNA directed primers (30 cycles were performed)

DNA source	Specific generation of amplicon	Inter-LINE PCR
Proteobacteria		
alpha-subclass		
Azospirillum brasiliense IBOE Sp7	+	n.d.
Ochrobactrum anthropi LMG 2136	+	n.d.
Sinorhizobium meliloti DSMZ 1981	+	n.d.
beta-subclass		
Alcaligenes faecalis DSMZ 30030^T	+	n.d.
Burkholderia cepacia DSMZ 7288	+	n.d.
Herbaspirillum seropedicae IBOE Z 67	+	n.d.
gamma subclass		
Pseudomonas fluorescens DSMZ 50090^T	+	n.d.
Agrobacterium tumefaciens DSMZ 30205^T	+	n.d.
Escherichia coli[a]	+	n.d.
Serratia marcescens DSM 30121^T	+	n.d.
CFB-group		
Cytophaga xantha DSMZ 3661	+	n.d.
Flavobacterium sp. DSMZ 1048	+	n.d.
High G+C bacteria (gram-positives)		
Arthrobacter citreus IBOE BI 90	+	n.d.
Corynebacterium glutamicum DSMZ 20300^T	+	n.d.
Micrococcus luteus[a]	+	n.d.
Nocardia carnea DSMZ 43397^T	+	n.d.
Streptomyces anulatus DSMZ 40361^T	+	n.d.
Clavibacter michiganensis sspp. DSMZ 1757, 7483, 46364	+	n.d.
Low G+C bacteria (gram-positives)		
Bacillus thuringiensis DSMZ 2046^T	+	n.d.
B. subtilis DSMZ 10^T	+	n.d.
B. cereus DSMZ 3101^T	+	n.d.
B. megaterium DSM 32^T	+	n.d.
B. licheniformis DSM 13^T	+	n.d.
Clostridium perfringens[a]	+	n.d.
Eukaryotic DNA		
calf thymus[a]	−	+
Mucor mucedo DSMZ 809	−	+
Aspergillus niger DSMZ 1988	−	+
Penicillium funiculosum DSMZ 1960	−	+
Irpex lacteus DSMZ 1183	−	+

n.d. = not determined.
Inter-LINE PCR = PCR with random primers.
[a] Genomic DNA, purchased from Sigma.

(DAPI) (Porter and Feig, 1980) as described by Wagner et al. (1993).

2.3. Extraction of DNA

DNA of bacteria and fungi, used for the evaluation of the specificity of the 16S rDNA targeted oligonucleotides (Table 1), was extracted by standard procedures (Marmur, 1961; Henrion et al., 1994) or purchased from Sigma (Deisenhofen, Germany). DNA of pure cultures for subsequent quantification was extracted by using the QIAamp Tissue Kit (Quiagen, Hilden, Germany). DNA of *Pseudomonas fluorescens* was extracted as recommended by the manufacturer.

In order to achieve an effective lysis, the procedure was slightly modified when *Bacillus* spp. were used: The pellets were resuspended in 1 ml of lysis buffer and transferred into 12 ml glass tubes each containing 2 g of 0.17–0.18 μm diameter glassbeads (Braun, Melsungen, Germany). During the 30-min incubation at 37 °C, the suspensions were homogenized three times for each 90 s (at the beginning, after 15 min and at the end of incubation) in a bead beater (Braun) at 2000 rpm. Soil DNA was extracted and purified by using the FastDNA SPIN Kit for Soil (Bio 101, Vista, USA) as recommended by the manufacturer.

2.4. Design of PCR primers and probes

The alignment for the development of the 16S rDNA specific oligonucleotide set was performed with 16S rDNA and 18S rDNA sequences of the different groups of organisms listed in Table 1, using the Genomatix DiAlign program (http://genomatix.gsf.de/cgi-bin/dialign/dialign.pl). Homologuos primer target regions were chosen to amplify DNA fragments of less than 300 bp. The probe target region was located within the amplified sequence as near as possible to one of the primers in order to enable an efficient hydrolysis of the probe during primer elongation by the polymerase. The oligonucleotides used in this study are described in Table 2.

2.5. Plasmid standards for absolute quantification

Plasmid standards were prepared as follows. The 16S rDNA fragments and the peptidase gene fragments were generated by conventional PCR using the corresponding primers to which sticky ends were added at the 5′ ends (the restriction recognition sequence for *Bam*HI plus overhang –CGC– to the forward primer and the restriction recognition sequence for *Xba*I plus overhang –GC– to the reverse primer). The obtained fragments were digested and cloned into the high copy number Triple Helix™ pHelix™ Vector 1(+) (Roche, Mannheim, Germany) and transformed into *Escherichia coli* JM 105. Purification of the plasmid DNA was performed with the Plasmid Midi Kit (Qiagen, Hilden, Germany). The quantity of the plasmid DNA was estimated by comparison of bands with those of Lambda DNA/*Eco*RI + *Hind*III Marker 3 (MBI Fermentas, Vilnius, Lithuania) on agarose gel after ethidium bromide staining. Tenfold dilutions were prepared and used as external standards in PCR. The origins of the cloned sequences are given in Table 2.

2.6. PCR

Amplification was carried out with the ABI 7700 Sequence Detection System (Perkin Elmer, Norwalk, CT, USA). The components for the 16S rDNA assay

Table 2
Oligonucleotides used as primers and TaqMan-probes for the quantification of 16S rRNA-genes and genes for alkaline metallopeptidases (*apr*), neutral metallopeptidases (*npr*) and serine peptidases (*sub*)

Oligonucleotide	Composition	Position (nt)[a]	Cloned target sequence fragment as standard
FP *16S rDNA*	5′-GGTAGTCYAYGCMSTAAACG-3′	799–818	*Escherichia coli* DSMZ 30083^T
probe 16S rDNA	FAM-TKCGCGTTGCDTCGAATTAAWCCAC-TAMRA	951–975	*16S rDNA* (263 bp)
RP *16S rDNA*	5′-GACARCCATGCASCACCTG-3′	1044–1063	
FP *apr* I	5′-TAYGGBTTCAAYTCCAAYAC-3′	808–827	*Pseudomonas fluorescens* DSMZ 50090^T
probe APR	FAM-ARCCVGAGAARTCVARGGTRTC-TAMRA	901–922	*apr* (194 bp)
RP *apr* II	5′-VGCGATSGAMACRTTRCC-3′	985–1002	
FP *npr* I	5′-GTDGAYGCHCAYTAYTAYGC-3′	214–233	*Bacillus cereus* DSMZ 3101^T
probe NPR	FAM-TAHAYCATYTGNKADCCRTTCCA-TAMRA	346–368	*npr* (233 bp)
RP *npr* II	5′-ACMGCATGBGTYADYTCATG-3′	437–446	
FP *sub* I	5′-ATGSAYRTTRYYAAYATGAG-3′	853–872	*Bacillus subtilis* DSMZ 10^T
probe SUB	FAM-TTGAHRTYDYKGCWCCWGGY-TAMRA	692–710	*sub* (319 bp)
RP *sub* II	5′-GWGWHGCCATNGAYGTWC-3′	1154–1171	

[a] Nucleotide position in the 16S rRNA gene of *E. coli* (Brosius et al., 1981), in the *apr* gene of *P. fluorescens* (NCBI AB013895), in the *npr* gene of *B. cereus* (NCBI M38910) and in the *sub* gene of *B. subtilis* (NCBI S51909). The peptidase targeting oligonucleotides are described in detail in Bach et al. (2001).

per 50 μl were: 5 μl of template DNA, 10 pmol of each primer, 7.5 pmol of TaqMan probe, 10 nmol of deoxynucleotide triphosphates, 5 μl of 10 × reaction buffer, 4.5 mM of $MgCl_2$ and 1.25 U of Ampli *Taq* Gold DNA Polymerase (Applied Biosytems) and H_2O ad 50 μl.

The components for the peptidase gene assays per 50 μl were: Template DNA: 5 μl; primers: 75 pmol of each *sub* I and *sub* II for subtilisins, 50 pmol of each primer *apr* I and *apr* II for alkaline metallopeptidases, 50 pmol of each primer *npr* I and *npr* II for neutral metallopeptidases; deoxynucleotide triphosphates: 10 nmol in each assay; 10 × SybrGreen PCR Buffer (Applied Biosystems): 5 μl in each assay; $MgCl_2$: 150 nmol for *sub* and *apr* and 175 nmol for *npr*; Ampli *Taq* Gold DNA polymerase (Applied Biosystems): 1.25 U for *sub* and *npr*, 1.00 U for *apr*; H_2O: ad 50 μl in each assay.

The PCR programs were as follows: One hold at 95 °C for 10 min for denaturation of DNA and activation of polymerase, 40 cycles of 95 °C for 20 s and 62 °C for 60 s when 16S rRNA genes were targets and 40 cycles of 95 °C for 20 s, 53 °C for 30 s and 72 °C for 30 s when peptidase genes were targets. Qualitative PCR (without the addition of probe) was performed in 50 μl volumes. For quantitative estimation for each replicate 80 μl volumes were prepared and aliquoted into two 38 μl measurement replicates, whose C_t values (Threshold-cycle) were averaged. The C_t value represents the cycle number at which the fluorescence signal crosses a fixed threshold. Since the SybrGreen detection assay is based on the intercalation of fluorescent SybrGreen into ds DNA (and also into PCR-by-products), specificity of PCR-products was checked on agarose gel.

2.7. Soils and sites

Soil samples originate from the Ap-horizon of an arable field in Scheyern, (Southern Germany), partially subjected to precision farming in the frame of the FAM research network on agroecosystems (http://www.fam.weihenstephan.de). The four variants are HC/LC—High yield/Low yield area fertilized according to conventional agricultural practice and HP/LP—High yield/Low yield area fertilized according to Precision farming techniques. The high yield area soil is a silty sandy loam, the low yield area is a silty loam. The field was cropped with maize (*Zea mays* L.) in 1999, followed by winter wheat (*Triticum aestivum* L.) in 2000.

3. Results and discussion

3.1. Specificity of the 16S rDNA targeted primers

The comparison of each designed oligonucleotide (Table 2) to known DNA sequences (EMBL, Release 66) using the Genomatix Matinspector program (http://genomatix.gsf.de/cgi-bin/matinspector/matinspector.pl) revealed that the detection system would be specific for eubacterial 16S rRNA genes and discriminate fungal as well as plastidial rRNA genes from either plants and animals. The Genomatix ModelInspector (http://genomatix.gsf.de/cgi-bin/gems/launch.pl) found a minimal length of 251 bp and a maximum length of 279 bp for the DNA sequence spanned by the two primers.

Specificity of the primers was tested empirically by PCR with genomic DNA of representives of different phylogenetic bacterial groups (Table 1) and DNA of eukaryotic organisms, omitting the TaqMan probe. Agarose gel electrophoresis of the PCR products and subsequent ethidium bromide staining revealed one single band of the expected length only for bacteria. No products were obtained when eukaryotic DNA was used as target. To make sure that lacking amplification of eukaryotic DNA was not the consequence of inhibition by the potential presence of contaminants, a PCR with random primers (Smida et al., 1996) was performed (Table 1). Resulting band patterns confirmed that DNA of all the samples were potentially amplifiable (data not shown).

3.2. Establishment of quantitative PCR assays

The sequences of primers and probes (Table 2) developed in this investigation and recently designed oligonucleotides for the amplification and detection of gene fragments of different bacterial peptidase classes (Bach et al., 2001) were applied to establish TaqMan-PCR assays for quantification. The components of the PCR mixes as given in Section 2.6 are the result of optimization with regard to threshold cycle and the level of the product plateau.

Table 3
Effectivity and reliability of the PCR quantification assays

TaqMan-assay	Standard curve	Correlation coefficient	Linear range (copies number)
16S rDNA$_{probe}$	$C_t = -3.837x + 39.892$	0.993	100–1 000 000
apr$_{SybrGreen}$	$C_t = -3.429x + 36.409$	0.996	200–2 000 000
npr$_{SybrGreen}$	$C_t = -3.472x + 35.332$	0.996	100–1 000 000
sub$_{SybrGreen}$	$C_t = -3.975x + 38.996$	0.994	10–100 000

apr = alkaline metallopeptidases, *npr* = neutral metallopeptidases, *sub* = serine peptidases, C_t = Threshold cycle, x = starting copy number.

3.2.1. Standard curves

The optimized PCR conditions were used to establish calibration curves with ten fold and onefold dilutions of the standard plasmid DNA ranging from 2×10^7 to 1×10^2 copies per μl (Table 3 and Fig. 1).

The function describing the relationship between C_t (threshold cycle) and x (log copy number) for the 16S rDNA assay has been calculated to be $C_t = -3.837x + 39.892$, with a correlation coefficient of 0.993.

Given the fact that at a 100% efficiency of the PCR, the C_t should decrease by 1.0 when the starting copy number is duplicated, the calculated delta C_t of 1.15 per onefold dilution of standard DNA suggests a very high efficiency of the presented PCR assay. Fig. 1 also shows the high reproducibility of the measurement at the machine level (superposition of the black symbols representing triplicate values).

For the peptidase gene fragments, very satisfying TaqMan results were obtained for the *apr* gene ($y = -3.170x + 37.324$, $R^2 = 0.982$; 20–2 000 000 copies) and for the *npr* gene ($y = -3.220x + 39.05$; $R^2 = 0.985$; 20–2 000 000 copies). For the *sub* gene, no reliable sigmoid PCR curves or even a standard curve could be generated (data not shown). Since neither *apr* nor the *npr* TaqMan assay were suitable for the quantification of more complex DNA, such as genomic DNA of pure cultures, PCR conditions were adapted to a SybrGreen detection, omitting the TaqMan probes. Standard curves given in Table 3 indicate that high reliability and effectiveness of PCR is also reached for the bacterial peptidase classes.

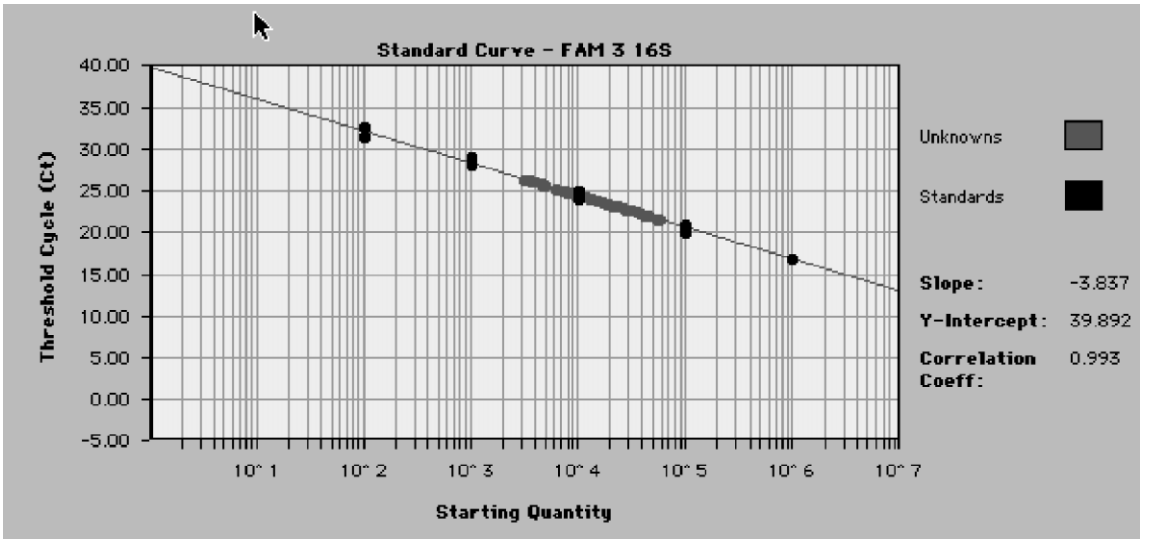

Fig. 1. Calibration curve plotting log starting copy numbers vs. C_t. The black symbols represent the triplicate PCR amplification of the standard DNA samples and grey symbols the duplicate PCR amplification of unknown genomic DNA. The Starting Quantity represents the initial number of target gene copies added to the PCR reaction mix.

Table 4
Inhibitory effect of DNA eluate on 16S rDNA targeting TaqMan-PCR

Source of DNA	Dilution in TE-buffer	C_t-value	Copies/μl
P. fluorescens[a]	none	17.9	3.11E+06
	1:10	19.31	1.26E+07
	1:100	22.67	1.38E+07
	1:1000	26.54	1.09E+07
soil HC[b]	1:10	40	0
	1:20	34.96	8.58E+02
	1:100	24.71	3.62E+06
	1:500	26.07	7.40E+06
	1:1000	27.97	4.25E+06
	1:5000	30.45	4.16E+06
soil LC[b]	1:10	40	0
	1:20	37.57	1.54E+02
	1:100	24.46	4.27E+06
	1:500	25.86	8.50E+06
	1:1000	27.01	8.00E+06
	1:5000	29.46	7.97E+06

[a] Extraction has been performed with the QIAamp Tissue Kit (Qiagen, Hiden, Germany).
[b] Extraction has been performed with the FastDNA SPIN Kit for Soil (Bio 101, Vista, USA).

3.2.2. Genomic DNA extracts

First PCR runs with genomic DNA indicated that inhibitory substances are coextracted during DNA extraction from pure cultures or from soil which might be responsible for inconsistent results. Genomic DNA of each strain was tested for its quality by relative quantification of gene copies in tenfold dilutions. Representative results are given in Table 4. The calculated copy concentrations show that DNA eluates from pure cultures and especially from soil are strongly inhibitory to PCR when it accounts for 10% of the total PCR volume. With soil DNA, no amplification is detected when 1% (1:10 dilution in TE buffer) is applied. An enhancement of PCR effectiveness could be observed when eluates were diluted up to 1:500. To eliminate inhibition and not to further raise the detection limit, 1:500 dilutions of the soil DNA eluates and 1:100 dilutions of the pure culture eluates were used as template DNA. The relatively high template volume of 10% was chosen to minimize pipetting inaccuracies.

3.3. Quantification of genes in pure culture

For the evaluation of the presented quantification assays, *P. fluorescens*, representing bacteria with alkaline metallopeptidases, *Bacillus cereus*, representing bacteria with neutral metallopeptidases and *B. subtilis*, representing bacteria with subtilisins were grown in liquid culture until the end of the exponential growth phase. Cell concentrations in terms of cfu, DAPI stainable bacteria and 16S rRNA and peptidase gene copy numbers are summarized in Table 5. The number of 16S rRNA genes per genome is reported to be 9 or probably 10 in *B. subtilis* (Loughney et al., 1982), 9 for *B. cereus* ATCC 10987 and 12 for *B. cereus* F0837/76 (Johansen et al., 1996) and 5 for *P. fluorescens* (Rainey and Bailey, 1996).

Comparing the total counts in terms of 16S rDNA copy numbers and DAPI counts, both based on the detection of DNA, and considering multiplicity of 16S rRNA operons (*rrn*), the TaqMan counts for *B. cereus* and *B. subtilis* are by factor 10 too low, suggesting an unsufficient lysis of bacterial cells or spores and incomplete release of DNA. For *P. fluorescens*, 16S rDNA copies are by factor 7.6 higher than DAPI counts, which is quite near to the theoretical value of 5. On the other hand, the cfu value representing the

Table 5
Cell concentration and concentration of gene copies in pure liquid cultures of bacteria

Strain	cfu/ml ($n=3$)	DAPI counts/ml ($n=10$)	16S rDNA copies/ml ($n=3$)	Reported number of *rrn* operons/genome	*apr* gene copies/ml ($n=3$)	*npr* gene copies/ml ($n=3$)	*sub* gene copies/ml ($n=3$)
Pseudomonas fluorescens DSMZ 50090^T	3.42E+08 (±2.23E+07)	1.13E+09 (±1.64E+08)	8.62E+09 (±1.41E+09)	5	1.37E+09 (±1.38E+08)	n.d.	n.d.
B. cereus DSMZ 3101^T	1.68E+08 (±2.84E+07)	2.16E+08 (±5.53E+07)	3.30E+08 (±7.31E+07)	9–12	n.d.	3.42E+07 (±4.92E+06)	n.d.
B. subtilis DSMZ 22^T	1.48E+09 (±3.24E+08)	5.90E+08 (±6.58E+07)	1.90E+08 (±7.87E+07)	10	n.d.	n.d.	3.47E+07 (±1.35E+06)

amount of culturable bacteria, is lower than DAPI counts and 16S rDNA copy numbers. Obviously, at the late state of the exponential growth phase, a large amount of bacteria has changed into a non-viable or viable but not culturable state. In contrast, the discrepancy between cfu and the DNA based values for *B. subtilis* may be due to the formation of spores, which are culturable, but neither stainable by DAPI, nor sufficiently disrupted for effective DNA extraction. Low DNA yields as a consequence of scarce lysis of spores of *Paenibacillus azotofixans* have also been reported by Rosado et al. (1996), who also applied three times bead beating. These authors could improve lysis efficacy by applying further bead beating treatments. However, the risk of shearing genomic DNA and damaging target sequences at continued beating (Miller et al., 1999) should be considered and the here presented approach for the quantification of targets would be quite suitable for such investigations.

For the quantification of peptidase genes, the same target DNA was used as for the estimation of 16S rRNA gene copies. Table 5 shows expected peptidase gene numbers in agreement with the 16S rDNA copy numbers. Assuming a copy number of one per genome for each peptidase, the number of genes for the alkaline peptidase in *P. fluorescens* (about 1 *apr* per 5 *rrn*), for neutral metallopeptidase in *B. cereus* (about 1 *npr* per 10 *rrn*) and for subtilisin of *B. subtilis* (about 1 *sub* per 10 *rrn*) correspond exactly to the values calculated from the 16S rDNA copy numbers per genome.

From these results, it is evident that for all three techniques applied for the quantification of bacteria their specific limitations have to be taken into account. However, two independent PCR assays led to nearly identical results which confirms the reliability and accurateness of the presented approaches for quantifying the genes present in a DNA extract. If rigid organisms such as *Bacillus*, vegetative cells or spores are investigated, further efforts have to be made to achieve a complete extraction of genomic DNA.

3.4. Quantification of 16S rRNA genes and peptidase genes in soil

3.4.1. Reliability

Reliability of five repeated soil DNA extractions with each three parallel TaqMan PCR quantifications (three separate 1:500 dilutions with subsequent measurement) is shown in Table 6. All DNA samples had been added to aliquotes of the same master mix and were measured in one TaqMan-PCR run. The standard deviations for the parallel quantifications of each DNA sample indicate a very high reproducibility of the measurement procedure itself. Standard deviation of the five repeated soil DNA extractions was 0.45 related to the mean values of the repetitions. Related to the standard curve in Fig. 1, the mean copy number in template DNA would be 2.82×10^3 per 5 μl with a standard deviation of 6.92×10^2. These results impressively show that both, DNA extraction by the FastDNA SPIN Kit for Soil (Bio 101, Vista, USA) as well as the TaqMan assay are highly reproducible.

3.4.2. Gene counts

The established assays have been applied for the investigation of total eubacteria and potentially proteolytic bacteria in soils subjected to different agricultural management practices. The sizes of detected gene populations, encoding 16S rRNA and subtilisins, as well as overall soil peptidase activity tend to reflect the intensity of farming management and soil fertility (Table 7). In the high yield area, where differentiated fertilization is more intensive than in the conventional variant, the values of all parameters are higher for the

Table 6
Reproducibility of DNA extraction from soil and the TaqMan quantification procedure. Results are given in terms of Threshold Cycle (C_t)

Feature	DNA-extraction-replicate					Mean	SD
	1	2	3	4	5		
Measurement 1	26.75	26.34	26.24	27.05	27.65	26.81	± 0.57
Measurement 2	26.89	26.16	26.29	26.76	27.44	26.71	± 0.51
Measurement 3	26.62	26.47	26.41	26.19	27.18	26.57	± 0.37
Mean	26.76	26.32	26.31	26.67	27.42	26.70	± 0.45
SD	± 0.13	± 0.16	± 0.09	± 0.44	± 0.24	–	–

SD = standard deviation.

Table 7
Bacterial gene concentrations in different soil samples as determined by real-time quantitative PCR

Management variant	Peptidase activity[a]	16S rRNA gene concentration[b]	*sub* gene concentration[b]
HC	191,65	3.51×10^8	1.72×10^6
HP	242,08	4.32×10^8	2.43×10^6
LC	124,58	2.48×10^8	1.96×10^6
LP	101,44	1.80×10^8	1.69×10^6

[a] Tyr [$\mu g \times g\ ds^{-1} \times 2\ h^{-1}$], activity was determined by the method of Ladd and Butler (1972).
[b] [gene g^{-1} dry soil].

HP, and at the low yield areas, where conventional fertilization is more intensive, values are higher in the LC variant. In general, the gene populations reveal much higher numbers than counts investigated by cultivation based methods such as MPN, which have been shown for arable fields to be between 10^6 and 10^7 g^{-1} for total bacteria and between 10^4 and 10^5 g^{-1} for proteolytic bacteria (Bach and Munch, 2000).

Since a direct DNA extraction was employed, the possible presence of DNA of dead cells or extracellular DNA in soil has to be taken into account. PCR based detection of DNA added to soil by Rosado et al. (1996) revealed that free DNA either is rapidly degraded or irreversibly adsorbed in soil or does not remain intact during lysis procedure. On the other hand, Recorbet et al. (1993) have reported an initial rapid decrease of *E. coli* target sequences after addition of cells to soil, but a persistence of remaining target sequences when population density had declined below the detection threshold of plate-counting. For the purpose of this study, we assumed that in soil, the number of target sequences associated with living cells exceeds the number of those which are not, since dead material is rapidly subjected to degradation by multiple transformation processes.

3.4.3. 16S rRNA gene multiplicity in mixed populations

Since the number of 16S rRNA genes per genome in prokaryotic organisms may vary from one single in *Rickettsia* (Anderson et al., 1995) to as many as 13 in *Clostridium beijerinckii* (Wilkinson and Young, 1995), only presumptions can be made about the size of the total bacterial population. There does not seem to be any phylogenetic conservation of the number of genes (Schmidt, 1998), but a correlation with the rate at which bacteria respond to resource availability. Klappenbach et al. (2001) assume that multiple copies of rRNA operons are required to achieve high growth rates as an ecological strategy, and found an average number of 5.5 copies in soil bacteria which rapidly form colonies upon exposure to complex media, and an average of 1.4 in soil bacteria only slowly forming colonies. So, even if underlying an average of 5.5 copies per genome, our TaqMan approach has detected genes of more bacteria than those detected by culture methods and usually described as viable but not culturable, and is suitable for the inclusion of this fraction into the investigation of changes in microbial populations.

3.4.4. 16S rDNA gene multiplicity and physiological status

Since these *rnn* operons are commonly found in the vicinity of the replication origin of the genome, an increase in the number of *rrn* loci in rapidly growing cells may result when DNA replication has started and cell division has not yet been completed. Okamoto et al. (1993) located 9 out of 10 rRNA gene regions between 0° and 90° on the chromosome map of *B. subtilis*. When the growth rate is faster than the DNA replication time, cells may inherit one multiforked chromosome or several replicating chromosomes (Trun, 1998). *E. coli* e.g., inherits one single chromosome when growth rates exceed 60 min (Trun and Gottesman, 1990). Maldonado et al. (1994) have studied the ploidy of *Azotobacter vinelandii* during growth and found up to 100 chromosome equivalents per cell during the late stationary phase. At slow growth rates, the numbers were similar to that of *E. coli*. However, in the natural habitate, growth rates are usually very low. For *Pseudomonas* spp. e.g., generation times of 5.2 h in the rhizosphere and 77 h in unplanted bulk soil have been reported by Bowen and Rovira (1976), so that polyploidy as a consequence of rapid growth may be negligeable depending on the sample under investigation.

3.5. Detection limit

The contaminant genomic DNA usually present in polymerase preparations in our experiments led to significant fluorescence in the no-template-controls after about 35 cycles (data not shown). This would

mean the presence of about 20 16S rRNA gene copies in each PCR tube with the PCR mix described above. Therefore, quantification would only be possible, if target gene concentration in template DNA exceeds 20 copies per 5 μl. Considering the sample dilutions, which were necessary to eliminate inhibition, the detection limit would be 2000 copies per 5 μl for liquid culture eluates and 10 000 copies per 5 μl for soil DNA eluates. Addition of BSA or DMSO, which were successful to improve classical PCR for peptidases without dilution (Bach et al., 2001), failed for the TaqMan assay as well as for the SybrGreen assays. Obviously, fluorescence based detection is quite sensitive to the addition of such compounds. We propose to introduce a magnetic capture-hybridization step (Jacobsen, 1995) by using the TaqMan probes presented in Table 2, which may eliminate inhibitory substances and may result in a relative enrichment of the target sequences by omitting background DNA.

4. Conclusion

The presented results clearly demonstrate that quantitative PCR by TaqMan as well as by SybrGreen detection with degenerate oligonucleotides are suitable for the quantification of bacteria and groups of bacteria in pure cultures or in complex habitates. To our knowledge, this is the first PCR based approach enabling an absolute quantification of an entire bacterial population and of a functional group of bacteria. The gene counts in soil samples indicate that also the non-culturable fraction of the soil community may be recorded, enabling the demonstration of shifts in bacterial community size. If certain species are to be detected, the number of rRNA operons per genome, which is conserved among all isolates, can be extracted from the Ribosomal RNA Operon Copy Number Database (www.http://rrndb.cme.msu.edu) or can be determined by a standard procedure for the determination proposed by Klappenbach et al. (2001).

When dealing with a highly complex mixture of bacteria from which neither the amount of *rrn* genes in the individual organisms nor their quantitative contribution to the total bacterial community is known, care should be taken when populations of different habitates are compared. By relating the amount of peptidase genes to the 16S rRNA gene population we will be able to study potential influences of farming management to an important group of organisms which is involved in nitrogen cycling by degrading peptidic compounds and making nitrogen available for living organisms. Further studies have to concentrate on the efficacy of DNA extraction and purity of template DNA.

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