Detection of *Saccharopolyspora rectivirgula* by Quantitative Real-Time PCR

JENNY SCHÄFER[1]*, PETER KÄMPFER[2] and UDO JÄCKEL[1]

[1]*Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, Nöldnerstrasse 40-42, 10317 Berlin, Germany;*
[2]*Institut für Angewandte Mikrobiologie, Justus-Liebig Universität Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany*

The thermophilic actinomycete species *Saccharopolyspora rectivirgula* has been associated with the exogen allergic alveolitis (EAA). EAA is caused by the inhalation of high amounts of airborne spores that can be found for example in environments of agricultural production, compost facilities, mushroom cultivation rooms, or rooms with technical air moistening. Because of the medical relevance of *S. rectivirgula*, a reliable detection system is needed. Therefore, a quantitative real-time polymerase chain reaction (qPCR) primer system was designed, targeting the 16S rRNA gene of the type strain *S. rectivirgula* DSM 43747[T] and six other *S. rectivirgula* reference strains. Our investigation showed that *S. rectivirgula* presumably own four operons of the 16S rRNA gene, which has to be considered for estimation of cell equivalents. Furthermore, the DNA recovery efficiency from these strains was tested in combination with bioaerosol or material sample as well as the influence of non-target DNA to the recovery rate. Results showed a recovery DNA efficiency of 7–55%. The recovery rate of DNA in a mixture with non-target DNA resulted in ~87%. In summary, a high amplification efficiency using real-time PCR was found, for which estimated concentrations revealed cell numbers of 2.7×10^5 cells m^{-3} in bioaerosol and 2.8×10^6 cells g^{-1} fw^{-1} in material samples from a duck house. The specificity of the new developed quantification system was shown by generation of two clone libraries from bioarosol samples, from a duck house, and from a composting plant. Totally, the results clearly show the specificity and practicability of the established qPCR assay for detection of *S. rectivirgula*.

INTRODUCTION

Saccharopolyspora rectivirgula (Krassilnikov and Agre, 1964; Korn-Wendisch *et al.*, 1989; Basonym: *Micropolyspora faeni*, Cross *et al.*, 1968 and *Faenia rectivirgula* Kurup and Agre, 1983) is an aerobic, thermophilic, Gram-positive, and filamentous bacterial strains belonging to the phylum *Actinobacteria*. *Saccharopolyspora rectivirgula* produces short chains of spores both on substrates and on aerial mycelia. The substrate mycelium ranged between

0.5 and 0.8 µm, the aerial mycelium between 0.8 and 1.2 µm, and spores vary between 0.7 and 1.5 µm in diameter (Kurup and Agre, 1983).

Saccharopolyspora rectivirgula was first isolated from soil and/or moldy hay in parallel (Lacey, 1990) and is well described as one causative agent of exogen allergic alveolitis (EAA, a type of hypersensitive pneumonitis, Corbaz *et al.*, 1963; Krassilnikov and Agre, 1964). EAA is an inflammation of the alveoli caused by hypersensitivity to inhaled organic dusts or in detail, by the inhalation of high amounts of different allergens, here of airborne spores of *S. rectivirgula*. The development of an EAA is dependent on predisposition of individuals as well as the nature, intensity, and

*Author to whom correspondence should be addressed.
Tel: 0049-30-515-48-4312; fax: 0049-30-515-48-4171;
e-mail: schaefer.jenny@baua.bund.de

duration of exposure. In Eastern Canada, e.g., *S. rectivirgula* was described to be most frequently responsible for 'farmer's lung disease', the classic form of EAA (Cormier *et al.*, 1985). However, high concentrations of *S. rectivirgula* spores were also found in compost facilities, mushroom cultivation, or rooms with technical air moistening (Pepys *et al.*, 1963; Lacey and Crook, 1988; Kutzner and Kempf, 1996; Danneberg and Driesel, 1999; Duchaine *et al.*, 1999). Because of the clinical relevance, a reliable detection system for *S. rectivirgula* is needed. Current detection methods for *S. rectivirgula* e.g. at working places are often based on cultivation-based approaches. However, identification of *S. rectivirgula* is difficult, especially when environmental samples are analyzed (Duchaine *et al.*, 1999). In addition, culture-based methods are time consuming and low in specificity and non-viable or dead bacterial cells, which can also cause allergic reactions, remain undetected. Hence, molecular approaches can be a useful alternative. Therefore, the aim of this study was the development of a quantitative real-time PCR (qPCR) assay for the specific detection of *S. rectivirgula* esp. in bioaerosols. Furthermore, influences on DNA extraction efficiencies should be analyzed to advert possibly underestimation of cell counts using molecular approach. Additionally, because quantification methods targeting the 16S rRNA gene possibly leading to an overestimation of analyzed cell counts, the amount of 16S rRNA gene copies in *S. rectivirgula* should be investigated.

MATERIALS AND METHODS

Bacterial strains and environmental samples

Testing the species-specific qPCR assay, we investigated seven *S. rectivirgula* strains obtained from the DSMZ (DSM 43747[T], DSM 43113, DSM 43114, DSM 43371, DSM 43755, DSM 43169 and DSM 43163). Twelve other *Saccharopolyspora* strains (DSM 44350[T], DSM 45019[T], DSM 45119[T], DSM 40517[T], DSM 44575[T], DSM 45244[T], DSM 43463[T], DSM 44795[T], DSM 43856[T], DSM 44771[T], DSM 44324[T] and DSM 44065[T]) also obtained from the DSMZ were used for optimization of qPCR protocol. All strains were either grown on the medium M65 (http://www.dsmz.de) or tryptone soy agar.

Mature compost material was obtained from two composting plants in Germany (anonymous) and straw material was obtained from one duck house in Germany (anonymous). Bioaerosol samples from different composting plants were collected by IPA (Institute for Prevention and Occupational Medicine of the German Social Accident Insurance) using a

personal sampling device as described earlier by Fallschissel *et al.* (2009). Bioaerosol samples from a duck house were taken by a stationary filtration system as described by Martin *et al.* (2009). Impacted cells were detached and homogenized from the employed polycarbonate filters (0.8 μm pore size, 37 mm in diameter, Whatman, Germany) into 10 ml NaCl 0.9% (w/v) using a stomacher (Stomacher 80 lab systems; Seward, London, UK) for 60 s and stored until usage at −20°C.

Extraction of DNA from bacterial strains and environmental samples

Genomic DNA from bacterial strains was extracted after disruption of cells by a 30-s bead-beating step (Precellys 24, Peqlab, Erlangen) with 1 g of 0.1 mm Zirconia beads (Carl Roth GmbH+Co, Karlsruhe) at maximum speed, with the GenElute™ Plant Genomic DNA Kit (Sigma) following the instructions of the manufacturer.

From the environmental samples, total DNA were extracted directly from 0.05 to 0.5 g material or from cells of 10 ml bioaerosol samples, which were concentrated by centrifugation (17 000 g) in a 2-ml reaction tube. The cell pellet was used for direct DNA extraction using the FastDNA®Spin Kit for soil (MP, Biomedicals) following the manufacturer's instructions. A negative control for DNA extraction, containing only the solutions of the extraction kit, was carried out to examine the purity of the solution of the extraction kit. The extracted DNA was used for further qPCR and cloning analyses.

Primer design

The nucleotide sequences of primer Sac-86f and Sac-183R, specific for 16S rRNA sequence fragments from *S. rectivirgula* species, were designed using the freeware programme Primrose 2.17 (Ashelford *et al.*, 2002), including the download of the current actual RDP database (http://rdp.cme.msu.edu/) as well as sequence information's from all *S. rectivirgula* strains, mentioned above. The developed primers Sac-86f: 5′-TGTGGTGGGGTGGATGAGT-3′ and Sac-183R: 5′-ACCATGCGGCAGAATGTCCT-3′ induce the amplification of a 16S rRNA gene fragment of ~100 bp. By submitting the nucleotide sequence to the PROBE MATCH algorithm of RDP (http://rdp.cme.msu.edu/index.jsp), the primer system initially was tested in silico for its specificity.

Quantitative real-time polymerase chain reaction

For preparation of quantification standards, fluorometric-quantified 16S rRNA PCR products (using

universal 16S rRNA primers, 27F/1492R, Lane, 1991), obtained from genomic *S. rectivirgula* (DSM 43747^T) DNA, were employed. For each concentration, the cycle threshold (C_T) value was plotted against the log value of corresponding target number. The calibration curve was generated by the iQ™5 software. Consequently, initial target copy numbers in the environmental samples were calculated as described by Martin *et al.* (2010). After optimization, the resulting qPCR conditions were initial denaturation at 98°C for 4 min, denaturation at 98°C for 1 min, annealing at 59.6°C for 10 s, and extension at 72°C for 10 s. To proof the occurrence of primer dimeres a step of 81°C for 10 s was added. The amplification was carried out at 50 cycles. The PCR was performed in a final volume of 20 µl, using the QuantiTect® SYBR® Green PCR Mix (Qiagen, Germany), with a primer concentration of 200 nM each primer in the iQ™5 Cycler (Biorad, Munich, Germany). For negative control, only SYBR® Green PCR Mix, primer solution and molecular grade water were analyzed. All samples, standards, and controls were analysed in triplicates.

16S rRNA operons: cloning analyses and southern hybridization of bacterial strains

To get detailed information about possibly differences in nucleotide sequences resulting from possibly multiple operons, 16S rRNA gene clone libraries were generated from all seven *S. rectivirgula* strains. Cloning analyses of the strains and subsequent sequencing of plasmid inserts were done by Agowa (Berlin, Germany) using the M13F primer (Invitrogen Corp., CA, USA).

Furthermore, the 16S rRNA operon copy number was estimated via southern hybridization according to the protocol from rrnDB database (http://ribosome.mmg.msu.edu/rrndb/about.php) and the digoxigenin (DIG)-High Prime Random Labeling and Detection Starter Kit II protocol (Roche, Molecular Biochemicals). First, we isolated genomic DNA from the *S. rectivirgula* type strain and digested the DNA with different restriction endonucleases (PstI, PvuII, SacI, XmiI, RsrII, SalI, Hind III, Fermentas). In the second step, the digested genomic DNA was separated via agarose gel electrophoresis. Subsequently, we transferred and immobilized the gel-separated genomic DNA to a (+)-charged nylon membrane (Roche, Molecular Biochemicals) and hybridized the membrane-bound genomic DNA with a DIG-labeled 16S rRNA gene probe. After specifically bounding of the probe, the immunological detection takes place with an alkaline phosphate-conjugated antibody specific to the DIG moiety on the DNA

probe. The hybridized DNA bands were detected with an alkaline phosphatase-activated chemiluminescent substrate.

DNA extraction efficiency and recovery rate using qPCR

The DNA extraction efficiency from *S. rectivirgula* as well as '*S. rectivirgulas* DNA' recovery efficiency were tested by spiking experiments. Beside the isolation of DNA from pure culture, *S. rectivirgula* cells were added to bioaerosol and material samples and furthermore, *S. rectivirgulas* DNA was added to DNA that was isolated from environmental samples. In the first assay, equal amounts of *S. rectivirgula* cultures [0.008 g fresh water (f.w.)] from different ages (3d, 7d, and 14d) were employed per DNA extraction assay, either as pure culture or spiked to bioaerosol samples out of a duck house or material samples (litter) from the same duck house each in triplicates. Although it is difficult due to the formation of filaments by this species for a rough estimation of applied cell numbers, we presume the fresh weight of *Escherichia coli* (9.5×10^{-13} g fw^{-1}) according to Madigan *et al.* (2001). Cell equivalents deployed in the assay were determined by calculation of 0.008 g divided by 9.5×10^{-13} g cells^{-1}, achieve an estimated amount of 8.42×10^9 cell equivalents. DNA extractions were done using the FastDNA®Spin Kit for soil (MP, Biomedicals) following the manufacturer's instructions. Amount of DNA was quantified fluorometrically (Qubit; Invitrogen). The amounts of *S. rectivirgula* cell equivalents were measured by real-time PCR approach (see above).

Values for *S. rectivirgula* originary present in the bioaerosol (2.7×10^5 cell equivalents) and material sample (2.8×10^6 cell equivalents) were considered by subtraction from values measured in the mixture with pure culture.

In the second approach, we investigated a potential inhibition of PCRs by non-target DNA. For this purpose, 1 µl of pure culture DNA (1 ng µl^{-1}, three stages of age) was mixed with 1 µl of bioaerosol DNA (0.5 ng µl^{-1}) each in triplicates. By real-time PCR assay from these mixtures, the 16S rRNA gene copy number of spiked *S. rectivirgula* DNA and the corresponding potential cell number were determined.

Cloning analyses of environmental samples and sequencing

Prior to quantitative analyses, the specificity of the developed qPCR system in environmental samples was investigated. Therefore, two positive PCR

products obtained from bioaerosol samples of (i) a duck house and (ii) a composting plant were analyzed by generation of two independent clone libraries (as described in Schäfer *et al.* 2010) and sequence analyses of plasmid inserts of 48 randomly chosen clones from each library. Cloning and sequencing analyses were done by Fraunhofer Institute (Aachen, Germany) using the M13F or M13R primer (Invitrogen Corp.).

Phylogenetic analyses

Similarity searches of all sequences out of all clone libraries against the NCBI database were carried out using BLAST search (http://www.ncbi.nlm.nih.gov/).

Multiple sequence alignment with type strains of the detected genera as well as genetic distance calculations (distance options according to the Kimura-2 model) of the data were also performed using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4.

RESULTS

16S rRNA operons in S. rectivirgula

Sequence analyses of all *S. rectivirgula* strains and clone inserts revealed differences both between and within the strains of *S. rectivirgula*, which may be explained by different 16S rRNA operons. About a fourth of all investigated 16S rRNA insert sequences of each strain could not be assigned to any known genus.

Highest sequence similarity using BLAST® search was detected to one uncultured bacterium found in a compost pile. These sequences however form one distinct cluster with high internal sequence similarity (>99.4%), which shows a clear indication for an unknown 16S rRNA operon in *S. rectivirgula*.

Southern hybridization revealed the presents of 3–5 bands per lane depending on used enzymes. In four lanes, which mean the digestion by PstI, PvuII, SacI, RsrII, four distinct bands were visible. Residual lanes showed ambiguous pattern with three and five bands [XmiI (5), SalI (3), Hind III (3)]. To estimate the equivalent cell number of *S. rectivirgula*, we primarly consider four 16S rRNA operons per *S. rectivirgula* genome. Based on 16S rRNA cloning analyses, however, we assume one operon of the 16S rRNA gene, which is not amplified by the new developed primer system. Therefore, finally, we calculated three operons per *S. rectivirgula* genome for qPCR analysis and calculation of cell equivalent units.

Quantitative real-time polymerase chain reaction

The analyses of the 16S rRNA fragment originating from the unexpected 16S rRNA operon showed that this fragment was not amplified (data not shown). Otherwise, a linear correlation ($r^2 = 0.99$) of C_T-values and corresponding target numbers was observed for concentrations between 10^3 and 10^8 targets μl^{-1}. The detection of *S. rectivirgula*, on the basis of $<10^3$ targets (35 cycles), was not possible because linear correlation failed. Whereas 16S rRNA genes from non-*S. rectivirgula* strains in general were not amplified, a weak unspecific gene amplification of *Saccharopolyspora cebuensis* could not be eliminated. Due to a very low amplification efficiency of *S. cebuensis* 16S rRNA gene, however, equal initial concentrations (1 ng μl^{-1}) of *S. rectivirgula* (DSM 43747^T) and *S. cebuensis* resulted in clear different quantification of 6.4×10^6 versus 4×10^3 cells μl^{-1}, respectively (data not shown). Additonally, until now, *S. cebuensis* was only isolated from a Philippine sponge (Pimentel-Elardo *et al.*, 2008) and seems to be not relevant in occupational environments with high exposure to airborne bacteria. The adequacy for the intended use of this PCR approach was indicated by melting curve analysis of PCR products and gel electrophoresis (bands showed the correct molecular size ~100 bp) of the amplicon (data not shown). Furthermore, the results revealed high amplification efficiency (~98%) of the 16S rRNA genes of all available strains of *S. rectivirgula* using the new designed primer system.

### DNA extraction efficiency and recovery rate

Firstly, the results showed DNA extraction efficiency from pure cultures between ~7% in 7-day old cultures, 19.5% in 3 days, and 55% in 14-day old cultures (Table 1, Column 4). The recovery rate from spiking experiments depends on age of the spiked cultures. Generally, the recovery rate was successful and varied between 60 and 100% in spiking experiments, respectively (Table 1, Columns 5 and 6). A worse recovery efficiency of 20% in material sample was found in spiking experiments with cells of a 14-day old culture (Table 1, Column 5). The investigated potential inhibition of PCRs by non-target DNA revealed a recovery between 70 and 100% (Fig. 1).

Specificity of developed primer system

Cloning analyses of PCR products gained with the new Primer system, from bioaerosol samples, of a duck house and a composting plant revealed that all obtained sequences ($n = 96$, each clone library 48) were most closely related (>99%) to 16S rRNA

Table 1. Employed amount of *Saccharopolyspora rectivirgula*, estimated cell number and cell number detected by qPCR and cell recovery in straw material or bioaerosol samples

Culture age (d)	Amount of culture in DNA extraction approach	Estimated equivalent cell number	Cell number detected by qPCR from pure culture (recovery %) ($n = 3$)	Cell number in straw material samples (recovery %, related to the pure culture recovery)	Cell number in bioaerosol samples (recovery %, related to the pure culture recovery)
3	0.008 g	8.42×10^9	$1.60 \times 10^9 \pm 2.42 \times 10^8$ (19.4%)	$1.24 \times 10^9 \pm 2.07 \times 10^8$ (77.6%)	$1.47 \times 10^9 \pm 4.65 \times 10^8$ (91.8%)
7	0.008 g	8.42×10^9	$5.67 \times 10^8 \pm 7.4 \times 10^7$ (6.7%)	$5.94 \times 10^8 \pm 1.14 \times 10^8$ (100%)	$5.61 \times 10^8 \pm 8.29 \times 10^7$ (99.0%)
14	0.008 g	8.42×10^9	$4.65 \times 10^9 \pm 3.19 \times 10^8$ (55.3%)	$9.28 \times 10^8 \pm 1.55 \times 10^8$ (19.9%)	$2.80 \times 10^9 \pm 3.61 \times 10^7$ (60.2%)
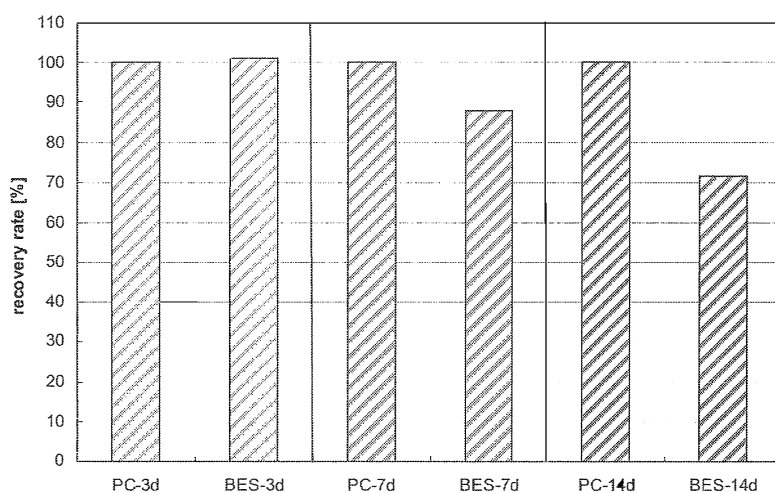
Fig. 1. Recovery of target DNA (1 ng μl^{-1}) from pure cultures (PC) with an age of 3d, 7d, and 14d in a mixture with DNA extracted from bioaerosols (BES). Values are means of $n = 9 \pm$ SD.

gene sequences from *S. rectivirgula*, verifying the adequacy of this PCR approach for the intended use.

Application in environmental samples

For testing the established qPCR protocol, we investigated bioaerosol and material samples from a composting plant and agricultural environment. Here, application of the method showed concentrations of *S. rectivirgula* between 2.7×10^5 and 1.0×10^7 estimated cell counts of *S. rectivirgula* in bioarosols and between 2.0×10^5 and 4.5×10^9 cell counts in material samples. Extended cell numbers of *S. rectivirgula* of 4.5×10^9 cells g^{-1} fw^{-1} were detected in mature compost and up to 1.0×10^7 cells m^{-3} in bioaerosol samples from composting plant (Table 2). Estimated cell numbers in straw material of a duck house amount 1.0×10^7 cells g^{-1} fw^{-1}. Bioaerosol samples out of the duck houses showed estimated cell numbers between 2.7 and 9.2×10^5 cells m^{-3}.

DISCUSSION

Current detection methods for *S. rectivirgula* based on its cultivation. Therefore, it is hardly to compare the few existing investigations with investigations of the present study. However, our results tend to result in clear higher *S. rectivirgula* concentrations. Lacey and Crook (1988), for example, detected *S. rectivirgula* together with *Thermoactinomyces* spp. in a concentration of 1.5×10^5 cfu m^{-3} air in mushroom farms. And Ranalli *et al.* (1999) detected up to 5.2×10^3 cfu m^{-3} air thermophilic *Actinomycetes* in diary barns, where they also detected similar amounts of thermophilic *Actinomycetes* in hay samples (3.3×10^3 g^{-1}). In contrast within the present study, the estimated amount of *S. rectivirgula* cells using qPCR assay was 1.0×10^7 cells per g^{-1} straw material and 2.8×10^6 cells per m^{-3} in bioaerosol samples from duck houses (Table 2). In general, this observation is in agreement with detected differences between culture-based and real-time

Table 2. Estimated cell counts of *Saccharopolyspora rectivirgula* per (gram per fresh water) or (m³) from different environmental samples and the share compared to the total cell count

Sample	Total cell count	Estimated cell counts *S. rectivirgula* (g fw) or (m³)	Share compared to the total cell count in %
Material: straw	n.d.	1.0×10^7	n.d.
ES-6.1-1	n.d.	2.8×10^6	n.d.
ES-6.4-1	n.d.	$.1.3 \times 10^6$	n.d.
ES-6.7-1	n.d.	2.0×10^5	n.d.
Compost Du	n.d.	4.5×10^9	n.d.
Compost Dra	n.d.	7.0×10^5	n.d.
Bioaerosol BES 071010-8/17	4.00×10^7	9.2×10^5	2.3
BES 6.1-1-3	3.13×10^7	2.7×10^5	0.85
BC 090806-24-Witz	1.60×10^8	$5,06 \times 10^5$	0.3
BC 090930-31-Gand	1.10×10^8	$2,35 \times 10^6$	2.1
BC 090916-16-Bohm	3.30×10^7	$1,01 \times 10^7$	30.7
BC 090930-30-Gand	1.20×10^7	$1,29 \times 10^6$	10.8
BC 090923-26-Nieh	2.30×10^6	$1,05 \times 10^6$	45.6
BC 090707-29 Hof	6.00×10^6	$4,26 \times 10^5$	7.0
BC 090923-27 Nieh	8.50×10^6	$5,78 \times 10^5$	6.7
BC 090909-39 Lemgo	3.90×10^6	$5,86 \times 10^5$	15.3

n.d., not detected; ES, straw from different duck houses including faeces; BES, bioaerosol from a duck house; BC, bioaerosol from a compost plant; total cell count was measured by DAPI (4',6-diamidino-2-phenylindol) staining according to Martin *et al.* (2010), estimated cell counts were detected by qPCR, values of qPCR are means of triplicates.

PCR-based quantifications (Fallschissel *et al.*, 2009). Reasons resulting in underestimation of the concentration using culture-based methods are manifold, e.g. microbes, which are viable but not culturable. These bacteria are viable but their metabolic activity is very low and therefore reproduction failed (Staley and Konopka, 1985; Heidelberg *et al.*, 1997; Zinder and Salyers, 2001; Oliver, 2005). Furthermore, desiccation and/or sampling procedure could result in loss of culti-vability. Especially, an air sampling by personal carried devices during a whole working day, which is the basic requirement for a precise exposure measurement, is not feasible in cultivation-based approaches because many bacteria are not resistant to sampling stress and desiccation (Marthi *et al.*, 1990; Potts 1994; Durand *et al.*, 2002). Here, qPCR permit the analysis of bioaer-osol samples that were collected over a period of several hours.

But even for these DNA-based methods, bias has been shown (Chandler, 1998; Polz and Cavanaugh, 1998; Acinas *et al.*, 2005) and methods generally should be used with caution. Therefore, our investigations should mention the limitations according to the DNA extraction efficiency and/or recovery. In particular, the loss of genomic DNA (up to 93%, Table 1, Column 4) in our study at the extraction procedure was comparable to those found earlier (Mumy and Findlay, 2004; Einen *et al.*, 2008;

Fallschissel *et al.*, 2009). In this context, an impact on DNA extraction efficiency was detected accord-ing to the age of the culture (Table 1). Here, may be (time of) sporulation and fragmentation of the culture influences DNA extraction efficiency be-cause of the difficulty of DNA extraction from spores. These findings basically show the difficulty in exact quantification of bacteria in environmental samples. However, in comparison to culture-based analyses of thermophilic *Actinomycetes*, qPCR show a clear improvement because a species-specific cul-tivation and a morphological differentiation to other thermophilic *Bacillus* spp. and *Geobacillus* spp. is hardly possible (Albrecht and Kämpfer, 2006). In consideration of the estimated loss of DNA by the isolation method, the real concentrations of *S. rectivirgula* in investigated samples seem to be 2- to 10-fold higher. A potential inhibition of PCR by co-extracted inhibitory substances or non-target DNA seems negligible because the recovery rate varied between 70 and 100% (Fig. 1).

Basically, a cell number can be deduced from PCR-based gene quantification. For this, the knowl-edge about number of target sequences for primer hybridization is most relevant. The primer system was used in the present study targeting the 16S rRNA gene sequence, which possibly occur multiple (Acinas *et al.*, 2004). Prior to quantification in

environmental samples, the 16S rRNA gene copy number was examined by southern hybridization. Depending on employed restriction enzymes, the pattern revealed operon numbers between three and five (mostly four bands). Pattern with five bands may be occurred by unspecific digestion of the used enzyme or unspecific hybridization of employed probes. Pattern with three bands may result from crude separation of DNA within the agarose gel. Because cloning analyses supporting the hypotheses of four operons (about a fourth of all investigated 16S rRNA insert sequences of all strains could not be assigned to the original sequence) within the present study, calculations of cell counts were made in presumption that *S. rectivirgula* exhibit four 16S rRNA operons. Finally, we calculated with three operons per *S. rectivirgula* genome in qPCR analysis and calculation of cell equivalent units because amplification of the untypical sequence failed. If 16S rRNA of *S. rectivirgula* is analyzed without a previous cloning analyses, this unexpected sequence was detectable as background sequence in chromatograms of 16S rRNA gene sequence analysis of all investigated *S. rectivirgula* strains (data not shown).

For crude calculation of 16S rRNA genes in environmental samples, a high specificity of the employed primer system is necessary because of high amounts of 16S rRNA gene sequences from different bacteria. Therefore, the specificity of the developed primer system was evidenced by cloning analyses from environmental samples, whereas all analyzed sequences showed high sequence similarities (>99.4%) to sequences from *S. rectivirgula*.

CONCLUSIONS

qPCR generally seems to be a potential method for the species or genus-specific quantification in bioaerosols (Makino *et al.*, 2001; Makino and Cheun 2003; Zeng *et al.*, 2006; Cayer *et al.*, 2007; Dutil *et al.*, 2007; Oppliger *et al.*, 2008, Fallschissel *et al.*, 2009, Martin *et al.*, 2010) and presumably, this method is suitable for standardization in occupational exposure measurements in future. With this study, we extend the currently existing real-time PCR approches, with a quantification protocol for the detection of airborne *S. rectivirgula*, a non-infectious but a well-known causative of extrinsic allergic alveolitis (synonym: farmer's lung disease).

REFERENCES

Acinas SG, Marcelino LA, Klepac-Ceraj V, Polz MF. (2004) Divergence and redundancy of 16S rRNA sequences in genomes with multiple rrn operons. Int J Syst Bacteriol; 186: 2629–35.

Acinas SG, Sarma-Rupavtarm R, Klepac-Ceraj V, Polz MF. (2005) PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. Appl Environ Microbiol; 71: 8966–9.

Albrecht A, Kämpfer P. (2006) Potential overestimation of the actinomycetes concentration by growth of thermophilic species of Bacillus and Geobacillus on selective media for thermophilic actinomycetes. Gef Reinhalt Luft; 9: 365–7.

Ashelford KE, Weightman AJ, Fry JC. (2002) PRIMROSE: a computer program for generating and estimating the phylogenetic range of 16S rRNA oligonucleotide probes and primers in conjunction with the RDP-II database. Nucleic Acids Res; 30: 3481–9.

Cayer MP, Veillette M, Pageau P *et al.* (2007) Identification of mycobacteria in peat moss processing plants: application of molecular biology approaches. Can J Microbiol; 53: 92–9.

Chandler DP. (1998) Redefining relativity: quantitative PCR at low template concentrations for industrial and environmental microbiology. J Ind Microbiol Biotech; 21: 128–40.

Corbaz R, Gregory PH, Lacey ME. (1963) Thermophilic and mesophilic actinomycetes in mouldy hay. J Gen Microbiol; 32: 449–55.

Cornier Y, Belanger J, Durand P. (1985) Factors influencing the development of serum precipitins to farmer's lung antigen in Quebec dairy farmers. Thorax; 40: 138–42.

Cross T, Maciver M, Lacey J. (1968) The thermophilic actinomycetes in mouldy hay: *Micropolyspora faeni* spnov. J Gen Microbiol; 50: 351–9.

Danneberg G, Driesel A. (1999) Mikroorganismen in der Arbeitsplatzatmosphäre—Aktinomyceten. editors, KAN-Bericht 13, 2nd edn. St. Augustin, FV: Verein zur Förderung der Arbeitssicherheit in Europa e.V.

Duchaine C, Meriaux A, Brochu G *et al.* (1999) *Saccharopolyspora rectivirgula* from Quebec dairy barns: application of simplified criteria for the identification of an agent responsible for farmer's lung disease. J Med Microbiol; 48: 173–80.

Durand KTH, Muilenberg ML, Burge HA, Seixas NS. (2002) Effect of sampling time on the culturability of airborne fungi and bacteria sampled by filtration. Ann Occup Hyg; 46: 113–8.

Dutil S, Veillette M, Mériaux A *et al.* (2007) Aerosolization of mycobacteria and legionellae during dental treatment: low exposure despite dental unit contamination. Environ Microbiol; 9: 2836–43.

Einen J, Thorseth IH, Ovreas L. (2008) Enumeration of Archaea and Bacteria in seafloor basalt using real-time quantitative PCR and fluorescence microscopy. FEMS Microbiol Lett; 282: 182–7.

Fallschissel K, Jäckel U, Kämpfer P. (2009) Direct detection of salmonella cells in the air of livestock stables by real-time PCR. Ann Occup Hyg; 53: 859–68.

Heidelberg JF, Shahamat M, Levin M *et al.* (1997) Effect of aerolization on culturability and viability of gram-negative bacteria. Appl Environ Microbiol; 63: 3585–8.

Korn-Wendisch F, Kempf A, Grund E *et al.* (1989) Transfer of *Faenia rectivirgula* Kurup and Agre 1983 to the Genus *Saccharopolyspora* Lacey and Goodfellow 1975, Elevation of *Saccharopolyspora hirsuta* subsp. *taberi* Labeda 1987 to Species Level, and Emended Description of the Genus *Saccharopolyspora*. Int J Syst Bacteriol; 39: 430–41.

Krassilnikov NA, Agre NS. (1964) On two new species of *Thermopolyspora*. Hindustan Antibiot Bull; 6: 97–107.

Kurup VP, Agre NS. (1983) Transfer of *Micropolyspora* rectivirgula (Krassilnikov and Agre 1964) Lechevalier, Lechevalier, and Becker 1966 to Faenia gen. nov. Int J Syst Bacteriol; 33: 663–5.

Kutzner HJ, Kempf A. (1996) Vorkommen von Actinomyceten in der Luft von Abfallbehandlungsanlagen, series WAR 92, 50. Darmstädter Seminar: Abfalltechnik, Hygiene in der Abfallwirtschaft, editor. Verein zur Förderung des Instituts WAR. TU Darmstadt, Germany: Institut für Wasserversorgung und Grundwasserschutz, Abwassertechnik, Abfalltechnik, Industrielle Stoffkreisläufe Raum und Infrastrukturplanung -TU Darmstadt. pp. 13–54.

Lacey J. (1990) Taxonomic history of actinomycete strains bearing the epithet *rectivirgula*: correct citation of authorities. Int J Syst Bacteriol; 40: 467–8.

Lacey J, Crook B. (1988) Fungal and actinomycete spores as pollutants of the workplace and occupational allergens. Ann Occup Hyg; 32: 515–33.

Lane DJ. (1991) 16S/23S rRNA sequencing. In Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. New York, NY: John Wiley & Sons, Inc; pp. 115–48.

Madigan MT, Martinko JM, Parker J. (2001) Brock Mikrobiologie. Heidelberg, Germany: Spektrum Akademischer Verlag.

Makino S, Cheun HI. (2003) Application of the real-time PCR for the detection of airborne microbial pathogens in reference to the anthrax spores. J Microbiol Methods; 53: 141–7.

Makino SI, Cheun HI, Watarai M *et al.* (2001) Detection of *anthrax* spores from the air by real-Time PCR. Lett Appl Microbiol; 33: 237–40.

Marthi B, Fieland VP, Walter M *et al.* (1990) Survival of bacteria during aerosolization. Appl Environ Microbiol; 56: 3463–7.

Martin E, Fallschissel K, Kämpfer P *et al.* (2010) Detection of Jeotgalicoccus spp. in poultry house air. Syst Appl Microbiol; 33: 188–92.

Martin E, Kämpfer P, Jäckel U. (2009) Quantification and identification of culturable airborne bacteria from duck houses. Ann Occup Hyg; 54: 217–27.

Mumy KL, Findlay RH. (2004) Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR. J Microbiol Methods; 57: 259–68.

Oliver JD. (2005) The viable but nonculturable state in bacteria. J Microbiol; 43: 93–100.

Oppliger A, Charrie N, Droz PO *et al.* (2008) Exposure to bioaerosols in poultry houses at different stages of fattening; use of real-time PCR for airborne bacterial quantification. Ann Occup Hyg; 52: 405–12.

Pepys J, Jenkins P, Festensteign AN *et al.* (1963) Farmer's Lung. Thermophilic actinomycetes as a source of 'farmer's lung hay' antigens. Lancet; 2: 607–11.

Pimentel-Elardo SM, Tiro LP, Grozdanov L *et al.* (2008) Saccharopolyspora cebuensis sp. nov., a novel actinomycete isolated from a Philippine sponge (Porifera). Int J Syst Evol Microbiol; 58: 628–32.

Polz MF, Cavanaugh CM. (1998) Bias in template-to-product ratios in multitemplate PCR. Appl Environ Microbiol; 64: 3724–30.

Potts M. (1994) Desiccation tolerance of prokaryotes. Microbiol Rev; 58: 755–805.

Ranalli G, Grazia L, Roggeri A. (1999) The influence of hay-packing techniques on the presence of Saccharopolyspora rectivirgula. J Appl Microbiol; 87: 359–65.

Schäfer J, Jäckel U, Kämpfer P. (2010) Analysis of *Actinobacteria* from mould-colonized water damaged building material. Syst Appl Microbiol; 33: 260–8.

Staley JT, Konopka A. (1985) Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu Rev Microbiol; 39: 321–46.

Zeng Q, Westermark S, Rasmuson-Lestander A *et al.* (2006) Detection and quantification of *Cladosporium* in aerosols by real-time PCR. J Environ Monit; 8: 153–60.

Zinder SH, Salyers AA. (2001) Microbial ecology-new directions, new importance. In Boone DR, Castenholz RW and Garrity GM, editors. Bergey's manual of systematic bacteriology. 2nd edn. New York, NY: Springer Verlag; pp. 101–9.