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TITLE: A Rapid and Specific Method for Quantifying *Streptomyces* Competitive Dynamics in Complex Soil Communities

RUNNING TITLE: Quantifying *Streptomyces* in soil by real-time PCR

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Abstract

Quantifying target microbial populations in complex communities remains a barrier to studying species interactions in soil environments. qPCR assays were developed for quantifying pathogenic *Streptomyces scabies* and antibiotic-producing *S. lavendulae* strains in complex soil communities. This assay will be useful for evaluating the competitive dynamics of streptomycetes in soil.

Introduction. *Streptomyces* are ubiquitous soil bacteria that are noted for their capacity to produce a vast array of bioactive compounds, including antibiotics (10). Antibiotic-mediated species interactions are believed to be important to *Streptomyces* fitness and plant disease biocontrol in soil, yet quantitative data on *Streptomyces* interactions in soil are limited. Moreover, because the impacts of one species on another can be mediated through interactions with other microbes in the community, detecting these impacts requires a sensitive and accurate method for quantifying the target populations within a complex community. Here, we describe a sensitive and specific assay that targets a short hyper-variable region of the 16S rRNA gene to distinguish among *Streptomyces* in complex soil communities. *Streptomyces* strains DL93 (*S. lavendulae*, an antibiotic-producer that is effective in plant disease biocontrol; 9) and DL87 (*S. scabies*, a plant pathogen) were studied in the present work. This approach has significant potential to shed light on the diversity and complexity of *Streptomyces* species interactions in soil.

Primer selection. A 324-bp segment of the 16S rRNA gene from over 400 *Streptomyces* strains from native prairie and agricultural soils in the Midwestern USA (4, unpublished data) and 14 additional representative Type *Streptomyces* strains retrieved from GenBank was evaluated using ClustalW (11). The aligned sequences spanned a 326 bp region from 54 bp to 380 bp of the *S. coelicolor* 16S rRNA (Y00411; 1), including the hypervariable γ-region (5). Previously the γ-region was found to account for approximately 30% of the variation among streptomycete 16S rRNA gene sequences (5). Nucleotide sequence variation was observed in species- and/or group-specific sequences (Supplemental Fig. 1), suggesting that the different species included in the alignment could be distinguished by PCR-based approaches in a species- and/or group-specific manner.

Primers were designed for detecting *S. lavendulae* strain DL93 and *S. scabies* strain DL87. Primers for the SYBR Green assay utilized species-specific forward primers and a single conserved reverse primer designed from the hypervariable region (Table 1, Supplemental Fig. 1). Primers for the TaqMan assay utilized species-specific forward and reverse primers and a single TaqMan MGB probe for detecting amplicons from multiple strains (Table 1, Supplemental Fig. 1). All primers and the probe were designed using Primer Express (Applied Biosystems, Foster City, CA). Primers were from Integrated DNA Technologies (Coralville, IA) and the probe was from Applied Biosystems.

Assay development. Initial experiments utilized primers designed for a SYBR Green-based assay. However, detection of strains in non-sterilized field soil was not achieved due to a high amount of non-specific background amplification, which was assumed to be from indigenous streptomycetes. This problem of non-specific amplification was eliminated by the Taqman-based assay.

For testing the sensitivity and specificity of the Taqman-based assay, DNA was extracted from cultures as described previously (3). Reactions (25 µl) consisted of 12.5 µl 2x TaqMan Universal PCR Master Mix (Applied Biosytems), 1 µl of each primer at 40 pmol/µl, 1.5 µl of the probe at 2.5 µM, and 5µl DNA. Separate reactions were done with strain DL87 and DL93 primer pairs. Reaction conditions consisted of a single cycle of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The lower limit of detection for both strains was 0.01 pg genomic DNA (Supplemental Fig. 2A, B). Based on the genome size of streptomycetes sequenced to date (2, 6, 7), 0.01 pg represents approximately one genome equivalent, indicating that the sensitivity of the TaqMan assay is equivalent to a single bacterial cell. Standard curves generated for both strains showed a linear response from 0.01 pg to 10 ng DNA (Supplemental Fig. 2C, D). The sensitivity and range of the TaqMan assay is similar to the lower level and range of detection of pathogenic *Streptomyces* strains obtained in real-time qPCR assays targeting the *txtAB* operon (8). Mixtures of each DNA were used in amplification reactions and no interference in accurate measurement of DNA from either strain was detected (Supplemental Table 1).

Specificity of the assay was tested with DNA from 15 *Streptomyces* strains commonly found in soil (4). Each reaction contained 10 ng of template DNA. The DL93 primers amplified a product detected by the probe with DNA from *S. lavendulae* OTU groups 1 and 5 (Table 2). Strain DL93 shares sequence identity with these strains in the targeted region of the 16S rRNA gene (Supplemental Fig. 1). The ability to detect multiple strains in two OTUs extends the capacity of the assay to follow the population dynamics of additional strains. Weak amplification occurred with *S. flavogriseus* DNA (Ct=21), which shares 10/14 nucleotides with the forward primer and 17/18 nucleotides with the reverse primer. The primers designed for strain DL87 were highly specific and only amplified DNA from strain DL87.

Field soil was inoculated with a 10-fold serial dilution series of strain DL87 or DL93 spores and total soil DNA was isolated with the PowerSoil DNA kit (MoBio, Carlsbad, CA) according to the manufacturer's protocol after sonication for 10 min in extraction buffer and two rounds of homogenization using the FastPrep FP120 (MP Biomedicals, Solon, OH). The modification was needed for detecting low densities of DL93. PCR reactions were done as described above using 10 ng DNA. No amplification was detected in assays using DNA from non-inoculated soil. Population estimates using for both strains were linear from 10^2 to 10^6 CFU/g soil (Supplemental Fig. 3).

Quantifying *Streptomyces* colonization and competitive dynamics in complex soil communities. We evaluated interactions of DL87 and DL93 by inoculating them alone and together to total combined densities of 10^6 CFU/g autoclaved soil or $5x10^5$ and $5x10^3$ CFU/g field soil. Soils were incubated in the dark at room temperature for 6 days. Each treatment was replicated three times. Culturable cell counts were determined by removing 1.0 g soil samples at 1 and 6 days post-inoculation, shaking them in10 ml sterile water for 1 hour at 4°C and dilution plating onto oatmeal agar. Dilution plating was not conducted for the non-sterile field soil samples, as it is impossible to differentiate DL87 and DL93 from other *Streptomyces* in complex mixtures. Samples (0.25 g) were removed from sterilized and field soil at the same times for DNA extraction and used in qPCR reactions to provide corresponding estimates of DL87 and DL93 densities.

In autoclaved soil, DL93 and DL87 population estimates were similar whether measured by qPCR or by dilution plating. Densities of DL87 were negatively impacted by DL93 at both time points (Fig. 1, $p<0.05$), reflecting the negative impacts of the antibiotic-producer on the establishment and growth of DL87 in autoclaved soil. In contrast, after 6 days DL93 populations were larger when co-inoculated with DL87 than when inoculated alone, suggesting a positive impact of DL87 on DL93 (Fig. 1). In field soil, DL93 also negatively impacted DL87 populations at each dose and time except after 1 day at the low inoculum dose (Fig. 2). However, no positive impact of DL87 on DL93 was observed, and after 1 day, at the low inoculum dose populations of DL93 were actually smallest in the presence of DL87.

These data suggest that significant competitive interactions occur between DL93 and DL87 in autoclaved soil and in the presence of a complex indigenous soil microbial community. However, the positive impact of DL87 on DL93 in autoclaved but not field soil indicates that the outcomes of microbial interactions in complex soil microbial communities may be different than in sterilized soil. The qPCR assay offers a powerful tool for elucidating microbial population and competitive dynamics under 'real-world' conditions.

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TABLE 1. Oligonucleotides used in this study

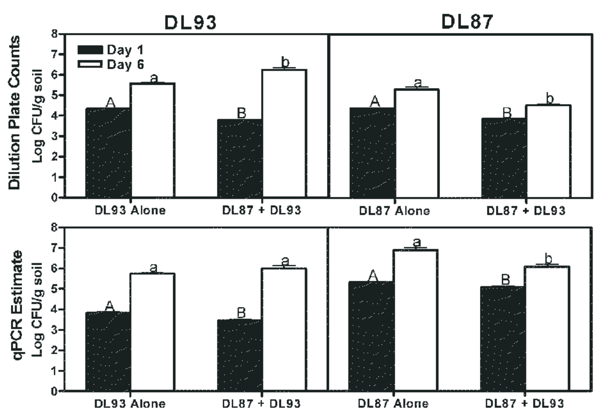
Target organism	Oligonucleotide	Sequence (5'-3')	Product size (bp)
S. scabies			
DL87	87FSYBR	GGTCTAATACCGGATACGACACTCT	164
	87/93RSYBR	GCTACCCGTCGTCGCCT	
	87taqF	CGGGCATCCGATGAGTGT	
	87taqR	GAGCCGTTACCTCACCAACAA	82
S. lavendulae			
DL93	93FSYBR	TCTAATACCGGATACCACTCCTG	161
	93taqF	GGATACCACTCCTGCCTGCAT	89
	93taqR	ATTACCCCACCAACAAGCTGAT	
Streptomyces sp.	Strep-probe	FAM-CGGTGAAGGATGAGC-MGB	

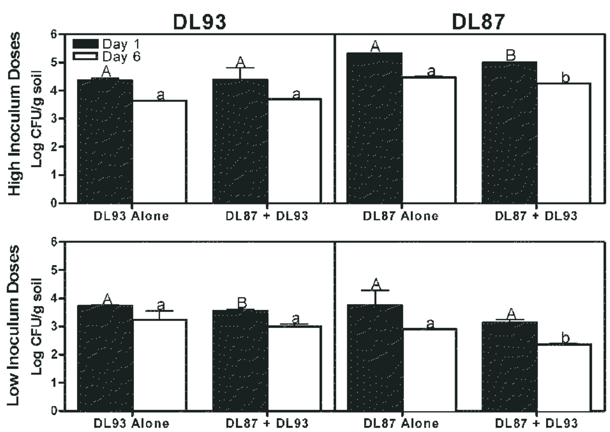
TABLE 2. Specificity of SYBR Green and TaqMan assays against common *Streptomyces* species found in Cedar Creek Ecosystem Science Reserve soil

				SYBR Green Assay		TaqMan Assay	
Species designation	Strain	Accession number	OTU[a]	DL87 primers	DL93 primers	DL87 primers	DL93 primers
S. flavogriseus	LK1234.1	AY465284.1	14	-[b]	+	-	+/-
S. lavendulae	LK1312.4	AY465295.1	1	-	+	-	+
S. lavendulae	LK1132.3	AY465223.1	1	-	+	-	+
S. lavendulae	LK1334.1	AY465333.1	1	-	+	-	+
S. lavendulae	LK1223.4	AY465263.1	5	-	+	-	+
S. lavendulae	LK1231.3	AY465271.1	5	-	+	-	+
S. lydicus	LK1111.4	AY465187.1	3	-	-	-	-
S. lydicus	LK1314.5	AY465301.1	3	-	-	-	-
S. lydicus	LK1133.5	AY465228.1	3	-	-	-	-
S. lydicus	LK1212.1	AY465237.1	3	-	-	-	-
S. olivochromogenes	LK1111.3	AY465186.1	2	-	-	-	-
S. olivochromogenes	LK1332.1	AY465324.1	2	-	-	-	-
S. olivochromogenes	LK1334.2	AY465334.1	2	-	-	-	-
S. rimosus	LK1124.1	AY465212.1	4	-	-	-	-
S. rimosus	LK1324.5	AY465318.1	4	-	-	-	-
S. scabies	DL87	AY277383.1		+	-	+	-
S. lavendulae	DL93	AY277380.1		-	+	-	+

[a] Operational taxonomic unit

[b] - indicates no product detected. + indicates specific amplicon detected. +/- indicates low amount of amplicon detected.


DL93
DL87
Day 1
Day 6
Dilution Plate Counts
Log CFU/g soil
qPCR Estimate
Log CFU/g soil
DL93 Alone
DL87 + DL93
DL87 Alone
DL87 + DL93



DL93
DL87
High Inoculum Doses
Low Inoculum Doses
Log CFU/g soil
Day 1
Day 6
DL93 Alone
DL87 + DL93
DL87 Alone
DL87 + DL93

Figure 1. Comparison of direct colony counts and qPCR for quantification of *Streptomyces* strains in sterile soil when inoculated alone and together at 1 and 6 days post-inoculation. Different letters of the same font within each figure indicate statistical significance at $p<0.05$.

Figure 2. Quantification of *Streptomyces* strains in field soil when inoculated alone and together at 1 and 6 days post-inoculation. Different letters of the same font within each figure indicate statistical significance at $p<0.05$.