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In silico Evaluation of Molecular Probes for Detection and Identification of *Ralstonia solanacearum* and *Clavibacter michiganensis* subsp. *sepedonicus*

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Summary

Ralstonia solanacerum and *Clavibacter michiganensis* subsp. *sepedonicus* are the two most relevant bacterial pathogens of potato for which a large number of molecular diagnostic methods using specific DNA sequences have been developed. About one hundred oligonucleotides have been described and thoroughly tested experimentally. After having compiled and evaluated all these primers and probes *in silico* to check their specificity, many discrepancies were found. A detailed analysis permitted the recognition of different possible reasons for such discrepancies: sequencing errors in public sequences, wrong supposed specificity (sometimes due to more recent sequences than the oligonucleotides being evaluated) or even typing errors in the oligonucleotides. Although this study is an exercise about *in silico* evaluation using two potato bacterial pathogens as a model, the conclusions reflect not only information useful for phytopathologists but, in a broader scope, draw the main situations that can be found during an evaluation of probes, which can be surely found in other scenarios.

Key words: specificity – primer – molecular diagnostic – oligonucleotide design

Introduction

Ralstonia solanacearum is a *Betaproteobacteria* that causes brown rot on potato and is also responsible of bacterial wilt on several hundred plant species worldwide [4, 19]. *Clavibacter michiganensis* subsp. *sepedonicus* belongs to the *Actinobacteria* and is the causal agent of potato ring rot [3].

As they are responsible of important economic losses, phytosanitary regulations have been established in most countries to control and prevent new outbreaks. In the European Union both are considered quarantine pathogens [5] for which special Directives have been published by the European Commission [3, 4]. Of special concern is the spread of these microorganisms by the dissemination of latently infected seed potato tubers and also contaminated water in the case of *R. solanacearum* [9, 16, 19]. Since latent infections cannot be detected by visual inspection of tuber lots, laboratory tests are required. Among them, the conventional approaches include serological methods as well as bioassays or isolation procedures [11, 13]. However, the latter methods are rather time-consuming. On the other hand, in the last ten years efforts to develop methods based on specific DNA sequences (PCR and its variations, FISH, DNA chips) for the detection of these two pathogens, have been intense and productive.

Meanwhile, the genomic era has brought a considerable number of new sequences available, making possible to review and redefine *in silico* the specificity of the oligonucleotides that have been described until now aimed at these two bacterial plant pathogens. To our knowledge this is the first review that studies these aspects in depth.

Abbreviations: BDB, blood disease bacterium, FISH, fluorescent *in situ* hybridization

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Materials and Methods

Sequences versus taxonomy

Sequences are the key element of the molecular methods mentioned above. One of the most basic rules, though sometimes overlooked, when searching for specific sequence stretches and for designing oligonucleotides is to define and separate those sequences that belong to the target group (species, subspecies, pathovar, biovar). This is not an obvious task since the information associated to a sequence (sequence annotation) might be incomplete, imprecise or even wrong.

Thanks to comparative 16S rDNA sequence studies we know that *R. solanacearum* forms a cluster encompassing three subclusters that have been named [41] division 1, subdivision 2a and subdivision 2b (the latter two form division 2). This distribution has been confirmed with phylogenies based on other genes [15]. Subdivision 2b contains two other plant pathogens: *Pseudomonas syzygii* and the blood disease bacterium (BDB). Very recently [44], *P. syzygii* has been reclassified as *Ralstonia syzygii*. Due to the proximity of this three organisms in phylogenetic terms, we will refer to them as *R. solanacearum sensu lato* (Rs).

C. michiganensis subsp. *sepedonicus* can be readily distinguished from the other four subspecies of *Clavibacter michiganensis* based on their 16S rDNA sequences [28]. However, the total number of sequences from other strains within this genus is relatively small.

Review of molecular probes

Tables 1 and 2 show a list of primers and probes that have been described for the detection or identification of *R. solanacearum* and *C. michiganensis* subsp. *sepedonicus*, respectively. For these (and other pathogens) ribosomal DNA, and especially 16S rDNA, is the target gene more commonly used for the search of specific DNA sequences. Not in vain it is also the gene sequence that has been obtained more often and from a larger number of strains depicting more accurately the group consensus and, to a certain extent, the intraspecies variability. However, it should be kept in mind that differences within a set of sequences do not necessarily reflect true variability since these differences could be due to errors, in particular during the amplification and sequencing reactions.

To illustrate this, at positions 94, 156 and 1026 (*E. coli* numbering) some of the available 16S rDNA sequences of *R. solanacearum* have an extra nucleotide (G, G and C, respectively) while others do not. This has been noted before although the differences were not considered to be of phylogenetic importance [8]. By comparing all these sequences among themselves and with those of related organisms, and taking into account their source, overall quality, conservation profile and other considerations, we have concluded that the above mentioned gaps might be sequencing errors.

In silico evaluation of the probes

Different software tools can be used to test the specificity *in silico* of a given sequence stretch. In general, it is possible to distinguish between local packages and on-line services. The first ones are generally suited for more refined searches, and usually can link very conveniently the results to other tools, but they have the disadvantage of requiring continuous updating of the sequences. To avoid this, many researches prefer algorithms to be used on-line such as BLAST – Basic Local Alignment Search Tool [1] and for this reason we chose such tool for this study. However, in the case of the 16S rRNA gene we also took advantage of the software package ARB [26] and its many utilities for sequence comparison, edition of alignments, search of patterns, etc.

Similarity searches for short nucleotide sequences are not likely to give the expected results using the default parameters on a BLASTn (nucleotide-nucleotide BLAST) query. In practical terms such searches will often fail to find significant matches. To make it easier to the users there is an optimized BLAST service for this purpose (called "search for short/near exact matches") available at the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) in which the word size is reduced to seven and the expect value is increased to 1,000. However, when the list of hits is too large (as it might be the case with 16S rDNA targeted oligonucleotides) we found it convenient to lower the expect value (to 10 or 1) in order to eliminate the lower-scoring hits from the report. Each report was subjected to careful examination, especially when unsuspected results were retrieved from the analyses. Although it can be very tedious, in many cases additional information regarding the source of the sequence (origin of the strain, phylogeny, biovar, race, and so on) that is missing from the sequence report has to be completed by other means.

This *in silico* analysis of primers and probes reported for the detection and identification of *R. solanacearum* and *C. michiganensis* subsp. *sepedonicus* confirms in many cases the specificity described in the original papers, but in some others important discrepancies have been found. We make also account of presumable sequence errors in the target sequences. In other cases we have detected that in order to be fully concordant with their targets some oligonucleotide sequences need some minor changes (affecting usually one single nucleotide) suggesting that probably an error was introduced. The results are discussed below and summarized in Tables 1 and 2.

Results

a) *Ralstonia solanacearum*

Among those that are listed in Table 1, OLI1 [38] is the oligonucleotide more often reflected in the literature. Of all the 16S rDNA sequences from Rs containing this target region (59 in total), 30 lack a G residue at position 94 (*E. coli* numbering) and are not retrieved performing a BLAST search. However, as it has been already discussed, we consider it an error despite the large number of sequences affected. This supports the specificity of this primer that has been tested and validated in many experimental studies, as the SMT project CT97-2179 [6].

With respect to OLI-2, its real specificity is not defined in the original work [12], where only one strain (BR-95-32, belonging to biovar 2) was tested. The *in silico* analysis demonstrates that it matches essentially against sequences of Rs division 2 in a region that has served for the design of other primers aimed to delineate divisions 1 and 2 in Rs (Fig. 1).

Because in their 16S rDNA sequences there are not many stretches specific for Rs (or its divisions), most PCR experiments (conventional PCR, nested PCR, multiplex PCR) have been designed using broad-range oligonucleotides such as Y2, JE-2, DIV1R, DIV2R or B [8, 12, 15, 38]. For example JE-2 matches mostly *Beta-* and *Deltaproteobacteria*. JE-2 together with two other 16S rDNA primers already commented (OLI1 and OLI-2), have been proposed for a co-operational polymerase chain reaction (Co-PCR) that has been successfully applied to detect *R. solanacearum* in water samples [9].

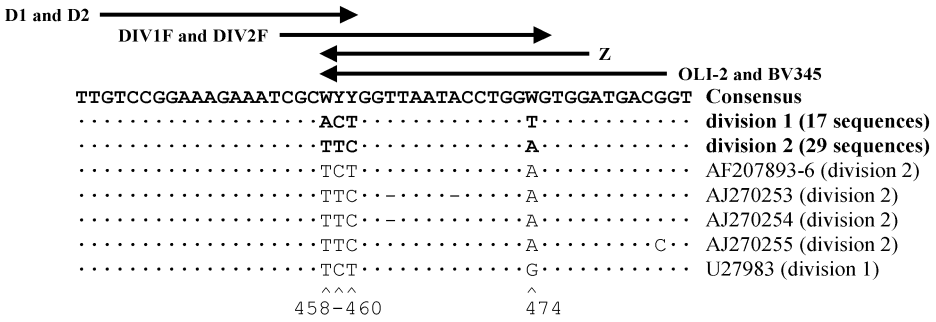

Fig. 1. Alignment of a region of the 16S rDNA of *R. solanacearum* for which several division-specific oligonucleotides (target sites shown as arrows) have been designed. Consensus sequences are in bold face. First line, Rs (ambiguous bases according to IUPAC code). Second and third lines are consensus sequences for division 1 and 2, respectively. Fourth to eighth lines correspond to non-consensus sequences. Dots indicate shared residues while dashes stand for gaps. Position (*E. coli* numbering) of the differential nucleotides shown at the bottom line.

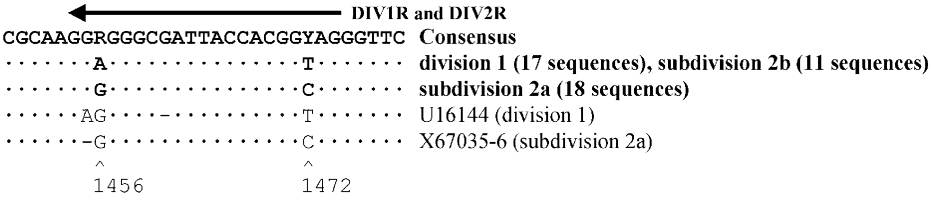

Fig. 2. Alignment of a region of the 16S rDNA of *R. solanacearum* showing the target site of primers DIV1R and DIV2R. First line (bold), consensus sequence (ambiguous residues according to IUPAC code). Second line (bold), consensus sequence for division 1 and subdivision 2b. Third line (bold), consensus sequence for subdivision 2a. Fourth and fifth lines, non-consensus sequences. Dots indicate shared residues while dashes stand for gaps. Position (*E. coli* numbering) of the differential nucleotides shown at the bottom line.

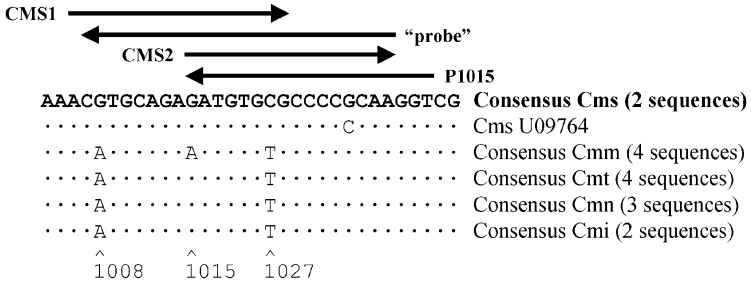

Fig. 3. Alignment of a region of the 16S rDNA for which several specific oligonucleotides (target sites shown as arrows) for *Clavibacter michiganensis* subsp. *sepedonicus* have been designed. First line (bold), consensus sequence for *C. michiganensis* subsp. *sepedonicus*. Second line, sequence U09764 that differs from consensus in one residue. Third to sixth lines, consensus sequences for the other subspecies of *C. michiganensis* (Cmm, *C. michiganensis* subsp. *michiganensis*, Cmt, *C. michiganensis* subsp. *tessellarius*, Cmn, *C. michiganensis* subsp. *nebraskensis*, Cmi, *C. michiganensis* subsp. *insidiosus*). Dots indicate shared residues. Position (*E. coli* numbering) of the differential nucleotides shown at the bottom line.

Primers DIV1R and DIV2R were described to be used together with forward primers DIV1F and DIV2F, respectively, to give a division-specific PCR product of 1019 bp [15]. However, when the match test is performed on these oligonucleotides, unexpected results are obtained since the delineation of divisions is not as published. DIV1F and DIV2F are specific for division 1 and 2, respectively (Fig. 1) whereas in the case of DIV1R and DIV2R, the first targets division 1 and subdivision 2b sequences and the second subdivision 2a (Fig. 2). This observation was not taken into account in the original paper [15].

Another discrepancy on specificity is that of primer D1 [8]. The *in silico* analysis shows that it matches perfectly not only with sequences of division 1 and some additional sequences of uncultured or unidentified organisms, but also with another from the recently described *Ralstonia insidiosa* [10] which includes isolates from the environment and human clinical samples. This illustrates very

Table 1. Oligonucleotide primers or probes described for the detection or identification of *R. solanacearum*. They are grouped according to the target gene and then chronologically.

Ref[a]	Name	Sequence (5′ → 3′)	Specificity[b]	Use[c]	Observations[d]
16S rRNA gene					
38	OLI1	GGGGGTAGCTTGCTACCTGCC	Rs	PCR (OLI1/Y2 = 287-288 bp)	
	Y2	CCCACTGCTGCCTCCCGTAGGAGT	*Non-specific*		
12	OLI-2	CGTCATCCACTCCAGGTATTAACCGAA	Rs	Nested PCR (OLI1/OLI-2 = not given,	OLI-2 is specific of Rs division 2
	JE-2	GTGGGGGATAACTAGTCGAAAGAC	*Non-specific*	and JE-2/Y2 = 172 bp)	OLI1/OLI-2 = 409 bp and JE-2/Y2 = 220 bp
15	DIV1F	CGCACTGGTTAATACCTGGTG	Rs 1	PCR (DIV1F/DIV1R = 1019 bp;	In addition to Rs 1, DIV1R matches Rs 2b
	DIV1R	CTACCGTGGTAATCGCCCTCC	Rs 1	DIV2F/DIV2R = 1019 bp)	as well as some *Betaproteobacteria*
	DIV2F	CGCTTCGGTTAATACCTGGAG	Rs 2		DIV2R matches Rs 2a and some
	DIV2R	CTGCCGTGGTAATCGCCCCCC	Rs 2		*Betaproteobacteria* (but not Rs 2b)
8	D1	GTCCGGAAAGAAATCGCACT	Rs 1	PCR (D1/B = 650 bp; D2/B = 650 bp;	D2 matches as well *Ralstonia insidiosa*
	D2	GTCCGGAAAGAAATCGCTTC	Rs 2	OLI1/Z = 403 bp)	and some other sequences
	Z	CCACTCCAGGTATTAACCGAA	Rs 2		
	B	GCGGGACTTAACCCAACATC	*Non-specific*		
39	BV345	CGTCATCCACACCAGGTATTAACCAGT	Rs 1	Multiplex PCR (OLI1/Y2= 292 bp; OLI1/BV345 = 409 bp)	
31	PS-1	AGTCGAACGGCAGCGGGGG	Rs	PCR (PS-1/PS-2 = 553 bp)	Both primers target many other
	PS-2	GGGGATTTCACATCGGTCTTGCA	Rs		*Betaproteobacteria*
45	RS-I-F	GCATGCCTTACACATGCAAGTC	Rs	Multiplex real-time fluorogenic PCR	RS-I-F and RS-II-R target many other
	RS-II-R	GGCACGTTCCGATGTATTACTCA	Rs	(RS-P is an internal probe)	*Betaproteobacteria*
	RS-P	AGCTTGCTACCTGCCGGCGAGTG	Rs		RS-I-F/RS-II-R = 93 bp
Internal Transcribed Spacer (ITS)					
15	ITSallF	TAGGCGTCCACACTTATCGGT	Rs	Multiplex PCR (ITSallF/PsALLR = 438 bp;	ITSDIV2F should be
	ITSDIV1F	GGCGGCGGAGAGCGATCT	Rs 1	ITSDIV1F/PsALLR = 312 bp;	**C**CAAACGCAAGCATCGAGTTTTC,
	ITSDIV2F	GCAAACGCAAGCATCGAGTTTTC	Rs 2	ITSDIV2F/PsALLR = 191 bp)	then it matches only Rs 2a
	PsALLR	TTCCAAGCGGTCTTTCGATCA	Rs + *Rp*		Information about the specificity of ITSallF and PsALLR is crisscrossed
39	ITSR	GCAGAGACTTCCACCTCCA	Rs 2	PCR (DIV2F/ITSR = 1246 bp)	ITSR should be GCAGAGACTTCCACC**G**TCCA
30	RS-1-F	ACTAACGAAGCAGAGATGCATTA	Rs	Multiplex PCR (RS-1-F/RS-1-R = 718 bp;	RS-1-F matches other *Betaproteobacteria*
	RS-1-R	CCCAGTCACGGCAGAGACT	Rs 2	RS-1-F/RS-3-R = 716 bp)	RS-1-R matches only subdivision 2a
	RS-3-R	TTCACGGCAAGATCGCTC	Rs 1		
16	RaSo41	CGTGCATTCTAGTTAGGCG	Rs	DNA array	RaSo41 matches Rs 2
	RaSo180	ACGGTGGAAGTCTCTGCC	Rs		RaSo180 matches Rs 2a
	RaSo299	CGCAAGCATCGAGTTTTC	Rs		RaSo299 matches Rs 2a (in part)
	RaSo333	ATTGCCAAGACGAGTAATAAC	Rs		RaSo333 matches Rs 2a (in part)
	RaSo405	ATGAGATGCTCGCAACAAC	Rs		RaSo405 matches Rs 2a (in part)
	RaSo460	GAGTGATCGAAAGACCGCT	Rs		
23S rRNA gene					
46	RSOLA	CACTTAGCCAATCTTAGGG	Rs	FISH	
	RSOLB	TTCGGTGACTGGCTTAGC	Rs		

42	RsolT2	TGCTGAATACATAGGCAAG	Rs non-Eur	PCR (RsolT2/RSOLB-R = 1046 bp;	Exceptions to the specificity of RsolT2
	RsolT3	CTGTTACTGAATTCATAGGTA	Rs Eur	RsolT3/RSOLB-R = 1046 bp)	and RsolT3 were found (see text)
	RSOLB-R	GCTAAGCCAGTCACCGAA	Rs		RsolT2/RSOLB-R = 1001 bp;
					RsolT3/RSOLB-R = 1005 bp
Polygalacturonase gene					
18	pehA#3	CAGCAGAACCCGCGCCTGATCCAG	Rs	PCR (pehA#3/pehA#6 = 504 bp)	Each primer has 1 mismatch to Rs GMI1000
	pehA#6	ATCGGACTTGATGCGCAGGCCGTT	Rs		
Endoglucanase gene					
15	PsyEndoF	GCCAGTGCACCGCCGCCTTC	*R. syzygii*	PCR (PsyEndoF/PsyEndoR = 395 bp)	PsyEndoF/PsyEndoR = 375 bp
	PsyEndoR	CGTTGCCGTAATGGCGCCCG			
Hypersensitive reaction and pathogenicity (*hrp*) gene					
33	RS30	GAAGAGGAACGACGGAAAGC	Rs	Nested PCR (RS30/RS31 = 1993 bp;	RS30 should be GAA**C**AGGAACGACGGAAAGC
	RS31	CGAACAGCCCACAGACAAGA	Rs	RS30a/RS31a = 256 bp;	RS31a matched also some *Xanthomonas* spp.
	RS30a	GGCGCTGGCGGTGAACATGG	Rs	RS30b/RS31b = 533 bp)	
	RS31a	CAACATCCTGGCCGGCATCGTG	Rs		
	RS30b	TCTTGCGCTCGCCCTTGATGTG	Rs		
	RS31b	CGACAGCAGCCGGCACCC	Rs		
Transposable element IS*1405*					
21	PS-IS-F	CGCAACGCTGGATGAACCC	Rs race 1	PCR (PS-IS-F/PS-IS-R = 1070 bp;	PS-IS-F and PS-IS-R showed 1 mismatch
	PS-IS-R	CAGACGATGCGAAGCCTGAC	Rs race 1	PS-IS-F/PS-IS-RA1 = 1181 bp;	each to *Rsol* GMI1000
	PS-IS-RA1	CACCCTAATCGGCACTAGCG	Rs A1	PS-IS-F/PS-IS-RB1 = 1256 bp)	
	PS-IS-RB1	CTCATGCTGACTGGCTACCC	Rs B1		
Unknown					
37	PS96-H	TCACCGAAGCCGAATCCGCGTCCATCAC	*Rsol*	PCR (PS96H/PS96I = 148 bp)	PS96-H and PS96-I have 3 and 2 mismatches
	PS96-I	AAGGTGTCGTCCAGCTCGAACCCGCC			to *Rsol* GMI1000, respectively
29	759	GTCGCCGTCAACTCACTTTCC	Rs	PCR (759/760 = 281 bp)	759 and 760 have 2 and 1 mismatches
	760	GTCGCCGTCAGCAATGCGGAATCG	Rs		to *Rsol* GMI1000, respectively
14	630	ATACAGAATTCGACCGGCACG	Rs bv 2	PCR (630/631 = 357 bp)	
	631	AATCACATGCAATTCGCCTACG	Rs bv 2		
22	BP4-R	GACGACATCATTTCCACCGGGCG	Rs	PCR (BP4-R/BP4-L = 1102 bp)	
	BP4-L	GGGTGAGATCGATTGTCTCCTTG	Rs		
45	B2-I-F	TGGCGCACTGCACTCAAC	Rs bv 2A	Multiplex real-time fluorogenic PCR	
	B2-II-R	AATCACATGCAATTCGCCTACG	Rs bv 2A	(B2-P is an internal probe)	
	B2-P	TTCAAGCCGAACACCTGCTGCAAG	Rs bv 2A		

[a] Reference.

[b] Names shortened as follows: Rs, *R. solanacearum* including *R. syzygii* and the BDB; Rs 1, *R. solanacearum* division 1; Rs 2, *R. solanacearum* division 2; *Rp*, *R. pickettii*; Rs non-Eur, non-European isolates of *R. solanacearum*; Rs Eur, European isolates of *R. solanacearum*; Rs race 1, *R. solanacearum* race 1; Rs A1, A1 subgroup of *R. solanacearum* race 1; Rs B1, B1 subgroup of *R. solanacearum* race 1; *Rsol*, *R. solanacearum sensu stricto*; Rs bv 2, *R. solanacearum* biovar 2; Rs bv 2A; *R. solanacearum* biovar 2A.

[c] Where applicable, amplicon sizes are given according to the original publication.

[d] Observations derived from the *in silico* analysis included here are corrections to the sequences, discrepancies on the specificity, and where applicable, deduced lengths of amplicons found to differ significantly from the previously reported ones (or not given).

Table 2. Oligonucleotide primers or probes described for the detection or identification of *C. michiganensis* subsp. *sepedonicus*. They are grouped according to the target gene and then chronologically.

Ref[a]	Name	Sequence (5' (3')	Specificity[b]	Use[c]	Observations[d]
16S rRNA gene					
28	'probe'	TTGCGGGGCGCACATCTCTGCACG	Cms	Dot blot	Some other target sequences found (*Cellulomonas* spp.)
20	CMR16F1	GTGATGTCAGAGCTTGCTCTGGCGGATC	Cm	Nested PCR	CMR16R1 and CMR16R2 target many
	CMR16R1	GTACGGCTACCTTGTTACGACTTAGT	Cm	(CMR16F1/CMR16R1;	High GC Gram-positive bacteria
	CMR16F2	CCCCGACTCTGGGATAACTGCTA	Cm	CMR16F2/CMR16R2)	Amplicon lengths are 1425 and 1275 bp, respectively
	CMR16R2	CGGTTAGGCCACTGGCTTCGGGTGTTACCGA	Cm		
24	P1015	ACCTTGGGGGGCGCACATC	Cms	FISH	Should be ACCTTG**C**GGGGCGCACATC Some other target sequences found (*Cellulomonas* spp.)
43	CMS1	ACGTGCAGAGATGTGCG	Cms	Northern blot	CMS2 should be GATGTGCGCCCC**G**CAA
	CMS2	GATGTGCGCCCCCCAA	Cms		For both some other target sequences found (mainly *Cellulomonas* spp.)
Internal Transcribed Spacer (ITS)					
23	Sp1f	CCTTGTGGGGTGGGAAAA	Cms	PCR (Sp1f/Sp5r = 215 bp)	
	Sp5r	TGTGATCCACCGGGTAAA	Cms		
32	PSA-1	CTCCTTGTGGGGTGGGAAAA	Cms	PCR (PSA-1/PSA-R = 502 bp)	
	PSA-R	TACTGAGATGTTTCACTTCCCC	*Non-specific*		
16	Cms181	GGGTGGGAAAATGGTCTG	Cms	DNA array	Cms181, Cms182 and Cms250A target also some eukaryotic sequences
	Cms182	GGTGGGAAAATGGTCTG	Cms		
	Cms250A	ACCAGACACACCAAAAGG	Cms		
	Cms250B	AACCAGACACACCAAAAGG	Cms		
	Cms250C	AAACCAGACACACCAAAAGG	Cms		
Plasmid pCS1					
36	CMS-6	CGCTCTCCCTCACCAGACTC	Cms	PCR (CMS-6/CMS-7 = 258 bp)	
	CMS-7	TCCCGTGCTTGCCTGCGTTG	Cms		
17	A47A	CACCCCTCGACTGCGAGAAACG	Cms		
	A47B	TCCTCCGAGACTTTCGGGACGC	Cms	PCR (A47A/A47B = 670 bp)	
40	CSRS-C	GGCCATGACGTTGGTGACAC	Cms + Cmi	PCR [inverted repeat] (CSRS-C/CSRS-C = 1054 bp)	
20	CMSIF1	TGTACTCGGCCATGACGTTGG	Cms	Nested PCR (CMSIF1/CMSIR1	CMSIR2 should be
	CMSIR1	TACTGGGTCATGACGTTGGT	Cms	= 1000 bp;	GATGAAGGGGTCAAGC**GC**GTC
	CMSIF2	TCCCACGGTAATGCTCGTCTG	Cms	CMSIF2/CMSIR2	CMSIF1/CMSIR1 = 1066 bp
	CMSIR2	GATGAAGGGGTCAAGCTGGTC	Cms	= 900 bp)	
Unknown					
27	CMS50F	GAGCGCGATAGAAGAGGAACTC	Cms	PCR (CMS50F/CMS50R = 193 bp;	
	CMS50R	TCCTGAGCAACGACAAGAAAA	Cms	CMS72F/CMS72R = 164 bp;	

	CMS72F	AGTTCGAGTTGATAGCAATCCGC	Cms	CMS85F/CMS85R = 205 bp)
	CMS72R	GTGTCTCGGATTCACGATCACC	Cms	
	CMS85F	AAGATCAGAAGCGACCCGCC	Cms	
	CMS85R	TCGCACAGCCAAATCCAGC	Cms	
35	Cms 50-2F	CGGAGCGCGATAGAAGAGGA	Cms	TaqMan PCR
	Cms 133R	GGCAGAGCATCGCTCAGTACC	Cms	
	Cms 50-53T	AAGGAAGTCGTCGGATGAAGATGCG	Cms	

Cms 50-2F/Cms 133R = 152 bp

[a] Reference.
[b] Names shortened as follows: Cms, *C. michiganensis* subsp. *sepedonicus*; Cm, *C. michiganensis*, including all five subspecies; Cmi, *C. michiganensis* subsp. *insidiosum*.
[c] Where applicable, amplicon sizes are given according to the original publication.
[d] Results of the *in silico* analysis included here are corrections to the sequences, discrepancies on the specificity, and where applicable, deduced lengths of amplicons found to differ significantly from the previously reported ones (or not given).

well how a new sequence can affect our perception of the predicted specificity of a certain probe and thereof the need for periodic reevaluation [2].

In the case of primers PS-1 and PS-2 [31] the lack of specificity is more evident since they allow the retrieval of more than 300 and 500 BLAST hits, respectively, corresponding to *Ralstonia* spp. and related organisms. Such primer pair combinations may still be used in conjunction with other primers or probes bearing the desired specificity, as is the case of the multiplex real-time fluorogenic assay of Weller et al. [45]. They employed a forward primer, RS-I-F, a reverse primer, RS-II-R, and a fluorogenic probe, RS-P. Both amplification primers exceed the maximum of 1000 hits (most of them *Betaproteobacteria*) that can be retrieved on a BLAST search, but RS-P yields only 25, all corresponding to Rs sequences.

In addition to the 16S rDNA targeted oligonucleotides, some others have been designed targeting the intergenic transcribed spacer (ITS) and the 23S rDNA. It is known that ITS reveals a higher sequence variability. This has been used to design a couple of division-specific primers, ITSDIV1F and ITSDIV2F [15] to be employed together with ITSallF and PsALLR in a multiplex PCR assay. The expected specificity of ITSallF (Rs) is not confirmed by the BLAST search since one *R. pickettii* sequence is retrieved together with 25 Rs hits. In the case of PsALLR, the reverse primer, only the 25 Rs hits are retrieved although the specificity was supposed to reach *R. pickettii* as well. Thus, it seems that this information was mixed up in the original publication [15]. One of the additional internal forward primers, ITSDIV1F, has a perfect match with nine *R. solanacearum* sequences (all belonging to division 1), while the other, ITSDIV2F, produces no perfect match and seems to be wrongly annotated. If the G residue at the 5′ end is corrected to C, then 12 *R. solanacearum* sequences (subdivision 2a) are retrieved as perfect matches.

Another important observation regarding oligonucleotides aimed at the ITS is that the sequence of ITSR, as shown in the original publication [39], retrieves only 11 hits and none of them corresponds to the sequences of *R. solanacearum*. The insertion of a G residue (Table 1) permitted to retrieve 14 hits, all of them corresponding to *R. solanacearum* subdivision 2a. Thus, we can assume that the sequence of primer ITSR was wrongly cited in the original paper and needs to be corrected.

Another couple of primers designed in that region are RS-1-R and RS-3-R [30]. Both are reverse primers designed to be used together with a forward primer, RS-1-F, that has its target on the 16S rDNA. This primer has been found in this study to be non-specific since it matches perfectly many other sequences of *Betaproteobacteria*. Therefore, the specificity of the PCR relies on the reverse primers (Table 1).

The set of probes of Fessehaie et al. [16] shows a gradient of specificities. Whereas RaSo460 matches all known ITS sequences of Rs, RaSo41 presents one mismatch to division 1 isolates, and the remaining five probes are specific for subdivision 2a or just part of it,

and one to seven mismatches to other sequences can be found. This could explain why some of the probes failed to hybridize with all the *R. solanacearum* strains used in that study [16]. Indeed, we have observed that the probes giving less positive results in that paper are also those found to have a more reduced specificity and vice versa. Sequencing of the ITS region of the strains used in their study would surely clarify this point.

RsolT2 and RsolT3, located in the same region but differing in some nucleotides, were designed upon 23S rDNA sequences to be specific for non-European and European isolates, respectively [42]. However, it is doubtful that the 23S rDNA sequences can support a geographical differentiation of the strains (at least it is not the case of the 16S rDNA). Indeed, two of the five sequences that have a perfect match to RsolT3 originated from non-European strains. So, it seems more plausible that RsolT2 and RsolT3 simply distinguish phylogenetic subclusters in the same way as the 16S rDNA divisions of Taghavi et al. [41]. Unfortunately, no correlation between the phylogenies of the two molecules can be established since there is only one strain (GMI1000) for which both the 16S and the 23S rDNAs are known [34]. There is evidence from studies with other groups of bacteria [7] that comparable results can be expected.

Oligonucleotides designed upon *R. solanacearum* sequences from other genes often produce very few BLAST hits simply because the number of strains used to sequence a particular gene is in most cases confined to a single one. As an example, pehA#3 and pehA#6 [18] present a perfect match to a sequence of *R. solanacearum* strain AW polygalacturonase A gene, but each one has one mismatch to the sequence of the same gene from *R. solanacearum* GMI1000 [34].

In the nested PCR assay of Poussier and Luisetti [33], primer RS30 – initially designed for *hrp* genes – seems to be wrongly annotated since it produces no BLAST hit. Once corrected (Table 1) three perfect matches are retrieved corresponding to *R. solanacearum* sequences of the *hrp* genes. For one of the internal primers, RS31a, nine additional non-target (*Xanthomonas* spp.) sequences of the *hrp* genes were obtained that matched perfectly. However, since it affects only one of the primers it has no implications on the specificity of the assay.

The oligonucleotide set PS-IS-F, PS-IS-R, PS-IS-RA1 and PS-IS-RB1 was designed to specifically detect race 1 isolates [21]. The first two oligonucleotides have a perfect match with the sequence of a transposase gene, IS*1405*, from *R. solanacearum* but this sequence is too short to include the target region of PS-IS-RA1 or PS-IS-RB1. Although *R. solanacearum* GMI1000 belongs to race 1, PS-IS-F and PS-IS-R can be located, at the right orientation and distance, on its genome sequence [32] but with one base mismatch each. On the other hand, PS-IS-RA1 and PS-IS-RB1 could not be located on that sequence.

PS96-H and PS96-I were the first specific primers described for *R. solanacearum* [37] but at the moment of their description the target gene was unknown. In this study, no perfect match could be obtained for these primers through a BLAST search. However, *R. solanacearum* GMI1000 megaplasmid [34], contains two stretches (in the right orientation and distance to yield a PCR product of the predicted size) but with three and two base differences to PS96-H and PS96-I, respectively. This region is part of a conserved hypothetical protein.

Primers 759 and 760 [29] were also designed for the detection of *R. solanacearum* using an unknown gene. Their evaluation revealed that primer 760 was almost coincident to two public sequences of *R. solanacearum* (only the 5' end differed) whereas primer 759 showed two differences to one of those sequences in *R. solanacearum* GMI1000 chromosome. The target gene codes for a probable type 2 peroxiredoxin protein.

Primers 630 and 631, specific of biovar 2 isolates [14], could not be located on the genome of strain GMI1000 (biovar 4) nor on any other public sequence. Nor could B2-I-F, B2-II-R (which is complementary and reverse to 631) or B2-P [45] and reported as specific of biovar 2A.

Finally, primers BP4-R and BP4-L [22] have perfect matches on *R. solanacearum* GMI1000 chromosome over a probable ribose-phosphate pyrophosphokinase, a hypothetical protein and a putative thymidine/pyrimidine nucleoside phosphorylase genes.

b) *Clavibacter michiganensis* subsp. *sepedonicus*

As can be seen in Table 2, the number of primers and probes described for this pathogen is less than for *R. solanacearum* (Table 1) and fewer target genes have been used. In contrast to *R. solanacearum*, the total number of sequences available from *C. michiganensis* subsp. *sepedonicus* is far more reduced and this was reflected in the results of the BLAST searches performed. Thus, for *C. michiganensis* subsp. *sepedonicus* there are only three 16S rDNA sequences and only one of them is almost complete. However, there is a genome sequencing project for this organism that is about to be completed (*Clavibacter michiganensis* Sequencing Group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/cm/). At the time of the writing of this manuscript, the theoretical coverage of reads was 99.98% and we have included in our studies the possibility of performing BLAST searches on this database to gain additional information. From those data, a full and corrected sequence of the 16S rDNA could be obtained. The consensus sequence of *C. michiganensis* subsp. *sepedonicus* contains three differential nucleotides. These are G at *E. coli* position 1008, G at position 1015 and C at position 1021 (Fig. 3). Not surprisingly this region has been used for a number of studies. One of them is the probe described by Mirza et al. [28] to which no particular name was given. However, although the probe can differentiate *C. michiganensis* subsp. *sepedonicus* from the other subspecies of *C. michiganensis*, it is not fully specific since we found six other sequences that showed a perfect match (three from *Cellulomonas flavigena*, one from *Cellulomonas iranensis* and two other not identified at the species level). Similar results were obtained for probe, P1015, designed to be used in FISH [23], and CMS1 and CMS2 to be used in northern blot [41]. For two of these oligonucleotides, P1015 and CMS2, a correc-

tion is proposed following our observations (Table 2). In the case of the nested PCR of Lee et al. [20] some discrepancies were also found for the reverse primers, CMR16R1 and CMR16R2, both of which showed a large list of BLAST hits (mostly high GC Gram-positive bacteria).

The oligonucleotides aimed at the ITS [16, 23, 32] yielded the expected specificity. Only for three of them, Cms 181, Cms 182 and Cms250A, a few other hits were found (mice and human sequences) that can be taken as random coincidences favored by the short length of these oligonucleotides (17 to 18 nucleotides).

A number of probes have been designed to target plasmid pCS1 [17, 20, 36, 40] which is present in all but one strains of *C. michiganensis* subsp. *sepedonicus* examined until now, either integrated in the chromosome or in episomic form [40]. For most of these probes the expected specificity is obtained in the *in silico* analysis. CSCR-C is derived from an inverted repeat region of the plasmid. It produced perfect matches against *Rhodococcus* sp., *C. michiganensis* subsp. *insidiosum* and *C. michiganensis* subsp. *sepedonicus* but only for the last two the sequence contains also a close target upstream in the appropriate orientation and distance, to make possible the PCR amplification of a 1054 bp fragment under non-maximal stringency conditions. For primer CMSIR2 a correction of its sequence is proposed (Table 2).

The remaining oligonucleotides from Table 2 have been located in genes from public databases giving the predicted specificity.

Conclusions

Diagnostic based on PCR and DNA-probe hybridization assays has had an ever increasing impact in Biology since their appearance. All these techniques rely on the recognition of specific, and discriminative, sequence signatures. The first of this attributes, specificity, can be inferred from the comparison of sequences and the second, discrimination, requires empirical testing. Some questions referring to both are discussed in the following lines.

What can we learn from an *in silico* analysis?

In an ideal situation a specific oligonucleotide will perfectly match only all available sequences known to belong to the organism (or group of organisms) for which it was designed. However many exceptions can be encountered contradicting our predictions. The researcher has to be aware that sequences are not free of errors and that they might be the cause of an unexpected BLAST result. Unfortunately, it is not always easy to judge whether a point mutation or insertion or deletion in a public sequence is significant or not. It also has to be considered that the information associated to a sequence (strain, origin, taxonomic status and so on) could be incomplete or wrong and thus lead to confusion in the process of defining the groups. If none of this occurs and yet the results of the *in silico* analysis are not as expected, the researcher has to redefine the specificity of the oligonucleotide or perform a new design. Another choice is the possibility of using a multi probe concept [25] to reduce problems resulting from identical target sites in thus far not known nucleic acids of non-target organisms.

In addition to the perfect matches, the reports of an *in silico* analysis may also contain sequences that were found to differ only slightly from the oligonucleotide being tested. Specially when these other sequences belong to organisms that may share the same habitat as the ones that we wish to detect it is important to include such organisms in the experimental tests to assess the discrimination power of the method.

This study also permitted us to calculate the predicted size of the amplicons for those oligonucleotides used as primers pairs in PCR. This information has been included in Tables 1 and 2 when it was not available in the original publication or when this value differed by more than 4 % from the reported one. Such deviations often occur when the size estimation is based on the band migration in an agarose gel and can be misleading for other researchers employing different conditions or molecular weight markers.

In silico analyses of previously described primers and probes

As the microbial genomes are being explored at such a rapid path, more and more sequences are being added to public databases spanning considerably the pool of possible targets to any given oligonucleotide. In many cases a perfect match occurs and the specificity of the probe/primer has to be defined in a broader sense. In other cases the situation is just the opposite and the real specificity can be more reduced than originally published, especially if the design was based on a short (sometimes just one) number of sequences which does not allow to predict the positional variability of the gene within the group of interest.

A reevaluation of specific oligonucleotides was never done before for *R. solanacerum* or *C. michiganensis* subsp. *sepedonicus*, and as discussed above interesting findings were found. Moreover, given their economic importance and the concern about their presence and spread, many efforts in detection systems, applied research, prospection and eradication programs are under continuous development. In this line, several EU projects have developed and validated protocols for the diagnostic of these bacteria that include PCR techniques using some of the primers included in this study.

For *R. solanacearum*, the primers sets recommended at the project SMT4-CT97-2179 [6] are OLI1/Y2 [38], PS-1/PS-2 [31] and RS-1-F/RS-1-R/RS-3-R [30]. With respect to the first pair, OLI1/Y2, the analysis of their sequences showed that OLI1 is specific for Rs (including *R. syzygii* and the BDB), while Y2 is non-specific. A similar situation is that of primers RS-1-F, RS-1-R and RS-3-R: the two reverse primers are specific for Rs subdivision 2a and Rs division 1, respectively, while the forward primer matches other *Betaproteobacteria*. In contrast, both sequences of primer pair PS-1/PS-2 were found to be non-specific for *R. solanacearum*. Their routine use in detec-

tion will show whether unexpected amplifications are likely to occur or not.

For *C. michiganensis* subsp. *sepedonicus*, primer pairs Cms 50-2F/Cms 133R [35] and PSA-1/PSA-R [32] have been proposed at the Diagpro protocol SMT4-CT98-2252. In this case, only primer PSA-R is non-specific since it matches sequences from the other *C. michiganensis* subspecies.

In summary, for this study we have compiled 97 specific primers and probes described for *R. solanacearum* and *C. michiganensis* subsp. *sepedonicus*. Their *in silico* analyses revealed that for 52 of them unexpected results and discrepancies were found affecting mostly the specificity. For 23 oligonucleotides, additional perfect matches to sequences from non-target organisms were obtained. On the contrary, 19 oligonucleotides showed an *in silico* specificity more reduced than originally described. We also believe to have found typing errors in the given sequence of six oligonucleotides.

All these observations should be taken into account for optimizing protocols and strategies for the detection and identification of *R. solanacearum* and *C. michiganensis* subsp. *sepedonicus*. In a broader sense we have shown the usefulness of an *in silico* re-evaluation of molecular probes.

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