Curr Microbiol (2013) 66:266–270
DOI 10.1007/s00284-012-0265-6

Putative and Unique Gene Sequence Utilization for the Design of Species Specific Probes as Modeled by *Lactobacillus plantarum*

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Received: 20 July 2012 / Accepted: 6 November 2012 / Published online: 25 November 2012

Abstract The concept of utilizing putative and unique gene sequences for the design of species specific probes was tested. The abundance profile of assigned functions within the *Lactobacillus plantarum* genome was used for the identification of the putative and unique gene sequence, *csh*. The targeted gene (*csh*) was used as the template for PCR amplification and construction of a non-radioactive DIG labeled probe. The *csh* derived probe aided in the preliminary and rapid identification of *L. plantarum* from mixed cultures by colony hybridization. The method described here for the rapid identification of *L. plantarum* can also be applied for the rapid detection of other bacteria if a unique gene sequence can be identified from its complete genome sequence.

Introduction

Species specific DNA probes have been extensively used for the screening, detection, enumeration, and identification of bacteria, yeasts, and viruses. Such DNA probes have been constructed targeting the ribosomal DNA intergenic spacers [13], the ribosomal DNA sequences [4, 8, 9], satellite DNA with highly repetitive DNA sequences [1], and well-characterized genes encoding functions relevant to specific metabolism or pathogenicity [6, 7, 14]. With the advent of chromosomal DNA sequencing and bioinformatics, it is currently possible to identify and utilize genes unique to a given genome as a species specific probe, without the necessity of extensive studies for the characterization of the selected gene. Although this approach requires the availability of a genome sequence, it could be potentially utilized for those organisms lacking a suitable region for the design of ribosomal DNA species specific probes. The absence of secondary structures on putative and unique gene sequences translates into fewer challenges for gene amplification and hybridization methods such as PCR, RT-PCR, and colony hybridization among others.

In theory and reality it is a challenge to achieve universal testing and absolute proof of the proposed putative and unique gene sequence rapid identification method. Considering this limitation, this research evaluates the practicality and appropriateness of the proposed putative and unique gene sequence approach for the identification, screening, and enumeration of *Lactobacillus plantarum* based upon the available genome sequence [10] for this organism. *L. plantarum* belongs to the *L. casei–Pediococcus* lineage along with *L. brevis* [11], genome sequences of which are also available allowing the completion of the appropriate analyses.

Materials and Methods

Strains and Growth Conditions

The strains utilized in this research are listed in Table 1. The lactic acid bacteria, staphylococci, and streptococci strains were transferred from frozen stocks to Lactobacilli

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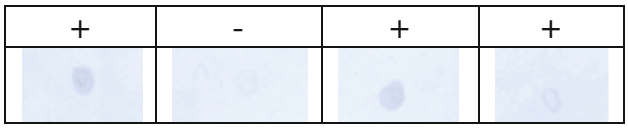

Fig. 1 Colonies that generated *colored spots* of any intensity, as shown in the *panel above*, were considered to specifically bind to the *csh* labeled probe during the hybridization process. Thus such colonies were counted as positive in Tables 1 and 2 (Color figure online)

deMan Rogosa and Sharpe agar (MRS) (Becton, Dickinson and Co., Sparks, MD). The *Listeria innocua* and *Enterococcus faecalis* strains were transferred from frozen stocks to Tryptic Soy Agar containing 1 % glucose (TSAG) (Becton, Dickinson and Co.). MRS plates were incubated at 30 °C, while TSAG plates were incubated at 37 °C.

Identification of the *L. plantarum* Putative and Unique Gene Sequence

The *L. plantarum* target gene was identified using the abundance profile search tool at the Joint Genome Institute-Integrated Microbial Genomes website (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=AbundanceProfiles&page=mergedForm) using the COG functional families. The abundance profile generated using the indicated tool represents the gene abundance count within a selected gene set.

The search was focused at identifying functions that are more abundant in *L. plantarum* than in any other organism listed in the database including archaea, bacteria, and eukaryota.

Amplification of *L. plantarum csh*, a Putative and Unique Gene Sequence

Primers for the putative and unique gene sequence, *csh* (accession number NP_786581), were designed using Lasergene PrimerSelect (DNASTAR, Inc., Madison, WI). The PCR amplification was performed using chromosomal DNA from *Lactobacillus plantarum* LA0219 as a template (10 μg/mL), which was extracted using a commercial genomic DNA purification kit (Promega Corporation, Madison, WI). The PCR mixtures contained Platinum PCR SuperMix (Invitrogen Corporation, Carlsbad, CA), chromosomal DNA, and primers CSHup (5′-TTAATTTTCCG CGCAACAAGT-3′) and CSHlo (5′-CTAAAAGCGAAG-TATGGCGTCAAA-3′) from Integrated DNA Technologies (San Diego, CA) added to a final concentration of 0.5 μM. The PCR cycle consisted of 4 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 57.5 °C, and 30 s at 72 °C, with a final extension step of 7 min at 72 °C and stored at 4 °C until used. *csh* was also amplified from the bacterial strains listed in Table 1. The 379 bp PCR products were purified (Qiagen PCR purification kit, Valencia, CA) and sequenced by Davis Sequencing (Davis, CA) using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA). The sequences obtained were analyzed by BLAST (Basic Local Alignment Search Tool) [2] in the GenBank database [5] to determine their homology and unique alignment with the *L. plantarum csh*.

The quality of the chromosomal DNA used for PCR was verified by amplifying a fragment of the 16S *rDNA* using primers Rd1 (5′-GTC TCA GTC CCA ATG TGG CC-3′), and Ru2 (5′-AGA GTT TGA TCC TGG CTC AG-3′) [3] (Sigma-Genosys, Saint Louis, MO) and following the protocol described above.

Generation of the *csh* Probe and Detection of *L. plantarum* by Hybridization

A fragment of the *csh* PCR product from *L. plantarum*, which was amplified using primers CSHup and CSH146 (5′-AGAAATGCTCAAGTATCGCCACAA-3′), was used to generate a digoxigenin (DIG) labeled probe. The 146 bp DIG labeled *csh* probe was generated with DIG High Prime (Roche Applied Science, Mannheim, Germany) using the random primed labeling technique and following the manufacturer's instructions. The DIG labeled *csh* probe was used for hybridization to 180–200 ng of chromosomal DNA isolated from the strains listed on Table 1. Chromosomal DNA was isolated as described previously and fixed to a positively charged nylon membrane by UV-crosslinking (Stratalinker UV Crosslinker, Stratagene, La Jolla, CA). Probe hybridization and detection were performed using the DIG High Prime Detection Starter Kit I (Roche Applied Science) and the DIG Wash and Block Buffer Set (Roche Applied Science) following the standard method described by the manufacturer. A hybridization temperature of 42 °C was applied.

Identification and Enumeration of *L. plantarum*

Cocktails of *L. brevis*, *Pediococcus pentosaceous*, *L. casei*, and *L. plantarum* were prepared by diluting stationary phase cultures to 10^6 CFU/mL with saline solution as described in Table 2. Cocktails were inoculated on MRS plates using a spiral plater (Spiral Biotech, Norwood, MA) for colony hybridization as described by Sambrook and Russell [12]. Briefly, colonies were transferred onto a nylon membrane by placing the membrane on the top surface of the MRS plates and pressing it with a spreading rod. Whatman 3MM filter paper disks soaked with either 10 % sodium dodecyl sulfate solution, denaturing solution (0.5 M NaOH and 1.5 M NaCl), neutralizing solution (0.5 M Tris–HCl, pH 8.0; 1.5 M NaCl), or 2 × SSC solution (0.3 M NaCl; 0.03 M sodium citrate) were sequentially placed in contact with the nylon membrane to achieve cell wall and membrane disruption and neutralization. Released chromosomal

Table 1 Microbial strains utilized in this study and *csh* fragment amplification and hybridization

Microorganisms	Strains	Source	16S *rDNA* fragment amplification by PCR	*csh* fragment amplification by PCR	*csh* derived probe hybridization
Lactobacillus plantarum	LA0086[a]	Unknown	+	+	+
	LA0219	Fermented cucumbers	+	+	+
	LA0406	Fresh cucumbers	+	+	+
	LA0403	Fresh cucumbers	+	+	+
	LA0516	Pickled/preserved cucumbers	+	+	+
	LA0402	Fresh cucumbers	+	+	+
	ATCC 14917	Pickled cabbage	+	+	+
	LA0405	Fresh cucumbers	+	+	+
	LA0086	Unknown	+	+	+
	LA0098	Unknown	+	+	+
	LA0309	Unknown	+	+	+
	ATCC BAA-793	Human oral cavity	+	+	+
Lactobacillus paraplantarum	ATCC 700211	Fermented beverages	+	−	−
Lactobacillus brevis	LA0025	Unknown	+	−	−
	ATCC 14869	Human feces	+	−	−
Lactobacillus acidophilus	LA0029	Unknown	+	−	−
Lactobacillus buchneri	ATCC 4005	Tomato pulp	+	−	−
Lactobacillus casei	NRRL B-441	Emmental cheese	+	−	−
Lactobacillus fermentum	ATCC 11739	Unknown	+	−	−
Pediococcus pentosaceous	ATCC 43201	Fermenting cucumbers	+	−	−
Lactococcus lactis	ATCC 13675	Dairy	+	−	−
Weisella confusa	ATCC 10881	Unknown	+	−	−
Leuconostoc mesenteroides	ATCC 8293	Fermenting olives	+	−	−
	ATCC 10880	Slime on root beer	+	−	−
	ATCC 10830	Unknown	+	−	−
	ATCC 23386	Sake starter	+	−	−
	ATCC 19254	Dairy	+	−	−
	ATCC 19255	Dairy	+	−	−
Leuconostoc paramesenteroides	ATCC 33313	Dairy	+	−	−
Listeria innocua	ATCC 33090	Cow brain	+	−	−
Staphylococcus aureus	B0031	Meat	+	−	−
Streptococcus agalactiae	LA0017	Unknown	+	−	−
Enterococcus faecalis	ATCC 19433	Unknown	+	−	−

ATCC American Type Culture Collection

[a] Strains with an LAXXX designation belong to the USDA-ARS Food Science Unit Culture Collection located in Raleigh, North Carolina, USA

DNA was fixed to nylon membranes by UV-crosslinking (Stratalinker UV Crosslinker, Stratagene). Hybridization was performed as described above.

Results and Discussion

Identifying a putative and unique gene sequence in any given organism is an intrinsically challenging task due to the lack of a universal database of genome sequences or an effective method for screening an infinite number of organisms. Recognizing this limitation, the approach taken in this research utilized the best model available to date, which is the most complete nucleic acid database. The question asked was whether there would be a unique function present in the genome of interest, *L. plantarum*. A list of seven clusters of orthologous groups (COG) functions were identified which were more abundant in *L. plantarum* WCSF1 than in any of the other organisms sequenced to date. The COG4814 function was the less abundant among the organisms

Table 2 Enumeration of *L. plantarum* colonies from mixed cultures containing *P. pentosaceous*, *L. brevis*, and *L. casei*

Membranes ID	Mixed culture ratio (%)		Total spot counts		
	L. plantarum ATCC 14917	*P. pentosaceous, L. brevis and/or L. casei* (1:1:1)	Estimated		Observed[a]
			Specific hybridization	Nonspecific hybridization	
A	100	0	56	–	56
B	75	25	36	40–48	31
C	50	50	26	34–50	27
D	25	75	10	19–38	11
E	0	100	0		

[a] Colonies that generated colored spots of any intensity as shown in Fig. 1, were considered as specific hybridization

represented in the database out of the seven functions identified. Function COG4814 is present in *Listeria* spp., *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., and all other lactic acid bacteria sequenced to date including lactobacilli, lactococci, leuconostoc, and pediococci. However, homology at the gene level is less than 70 % among the corresponding multiple genera genomes. All the nine gene sequences in *L. plantarum* linked to the COG4814 function encode for uncharacterized proteins with an alpha/beta hydrolase fold to which a cell surface hydrolase function was assigned. The nine *L. plantarum* gene sequences are 80 % similar, thus dissimilar regions on the selected *L. plantarum csh* were favored for primer design.

As predicted by the abundance profile analysis of the available genome sequences, it was possible to amplify the *csh* putative and unique gene sequence from all the *L. plantarum* strains screened. Identity of the PCR products was confirmed by sequencing. The similarity observed between the sequenced PCR products and the *L. plantarum csh* was of at least 99 %. Similarly, the *L. plantarum csh* was not amplified from members of the *L. casei–Pediococcus* lineage, which includes *L. casei, P. pentosaceus,* and *L. brevis* [11], or from the non-target strains tested, which harbor similar genes. Moreover, the results from the chromosomal DNA hybridization analysis agreed with the expected results further confirming the uniqueness of the *L. plantarum csh* (Table 2). When a filter membrane containing chromosomal DNA from a mixed culture of *L. plantarum* and other lactic acid bacteria was probed with a labeled *csh* fragment, no cross-hybridization was observed (Table 2).

Availability of a putative and unique gene sequence derived species specific probe could be potentially applied for the identification of *L. plantarum* in mixed cultures or from a pool of unknown isolates, and the detection of this bacterium in fermented, fresh, or spoiled foods, food ingredients, feces, plant material, and clinical samples among other sources. This approach to species identification could be applied to any microorganism for which there are complete genomes sequences for both the target organism and some closely related organisms.

In the possible event that a putative and unique gene sequence is present in organisms other than the one of choice, further manipulations, such as an increase in the stringency of the methods utilized, may be applied to achieve the unique identification desired. A combination of probes constructed with fragments of multiple unique genes in the microorganism of choice may be utilized to statistically reduce the chances of not identifying a given member of the targeted species lacking the selected gene. This approach would additionally strengthen the identification process and eliminate the necessity for further phenotypic and/or biochemical testing.

Acknowledgments The author thanks Ms. Ashlee Webber at the USDA-ARS, SAA Food Science Research Unit located in Raleigh, NC for technical assistance and Ms. Sandra Parker for excellent secretarial assistance.

References

1. Abad P (2000) Satellite DNA used as a species-specific probe for identification of *Bursaphelenchus xylophilus*. OEPP/EPPO Bulletin 30:571–574
2. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
3. Barrangou R, Yoon S-S, Breidt F, Fleming HP, Klaenhammer TR (2002) Identification and characterization of *Leuconostoc fallax* strains isolated from an industrial sauerkraut fermentation. Appl Environ Microbiol 68:2877–2884
4. Ben Amor K, Vaughan EE, de Vos WM (2007) Advanced molecular tools for the identification of lactic acid bacteria. J Nutr 137:741S–747S
5. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL (2006) GenBank. Nucl Acid Res 34(Database issue): D16–D20
6. Datta AR, Wentz BA, Hill WE (1987) Detection of hemolytic *Listeria monocytogenes* by using DNA colony hybridization. Appl Environ Microbiol 53:2256–2259
7. Ganesan K, Banerjee A, Datta A (1991) Molecular cloning of the secretory acid proteinase gene from *Candida albicans* and its use as a species specific probe. Infect Immun 59:2972–2977

8. Hyung PY, Park HKY, Kang BC, Song ES, Jang HJ, Kim CM (2006) Development of genus- and species-specific probe design system for pathogen detection based on 23S rDNA. J Microbiol Biotechnol 16:740–747
9. Kerkhof L (1994) A species specific probe and a PCR assay for the marine bacterium, *Pseudomonas stutzeri* strain *Zobell*. Microb Ecol 27:201–212
10. Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, Tarchini R, Peters SA, Sandbrink HM, Fiers MWEJ, Stiekema W, Klein Lankhorst RM, Bron PA, Hoffer SM, Nierop Groot MN, Kerkhoven R, de Vries M, Ursing B, deVos WM, Siezen RJ (2003) Complete genome sequence of *Lactobacillus plantarum* WCSF1. PNAS 100:1990–1995
11. Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee JH, Díaz-Muñiz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'Sullivan D, Steele J, Unlu G, Saier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D (2006) Comparative genomics of the lactic acid bacteria. PNAS 103:15611–15616
12. Sambrook J, Russell DW (eds) (2000) Molecular cloning, a laboratory manual, 3rd edn, Vol 1. Cold Spring Harbor Laboratory Press, Woodbury, pp 129–138
13. Schurko AM, Mendoza L, de Cock AW, Bedard JE, Klassen GR (2004) Development of a species specific probe for *Pythium insidiosum* and the diagnosis of pythiosis. J Clin Microbiol 42:2408–2411
14. Wright AC, Miceli GA, Landry WL, Christy JB, Watkins WD, Morris JG Jr (1993) Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. Appl Environ Microbiol 59:541–546