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SHORT COMMUNICATION

Specific PCR to identify the heavy-metal-resistant bacterium *Cupriavidus metallidurans*

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Abstract The aim of this study is to develop a polymerase chain reaction (PCR) assay for rapid detection of *Cupriavidus metallidurans*. PCR primers targeting the *Signal transduction histidine kinase* gene were designed and designated Cm-F1/Cm-R1. Strains of *C. metallidurans* were positively identified. The size of the PCR products was 437 bp, as expected. This PCR method enables monitoring of industrial, environmental and clinical sources for presence of *C. metallidurans*.

Keywords *Cupriavidus metallidurans* · PCR primers

Introduction

The mesophilic, Gram-negative, facultative chemolithoautotrophic β-proteobacterium *Cupriavidus* (formerly *Ralstonia* and *Wautersia*) *metallidurans* was isolated in 1976 as a cadmium-resistant and hydrogen-oxidising pseudomonad from a decantation tank at a metal processing factory in Liege, Belgium [10]. This strain, later called CH34, was found to be highly resistant to Zn^{2+}, Cd^{2+} and Co^{2+}; the extrachromosomal genetic determinants conferring this resistance were transferable to related bacteria [7]. The bacterium was found to prevail in industrial anthropogenic biotopes such as metallurgic wastes [11]. CH34 is being considered for bioremediation in areas of selenite pollution, where it has the ability to reduce the element and to store it as selenium granules [13]. Other potential industrial applications proposed for *C. metallidurans* include its use in biosensors for monitoring the concentration of bioavailable heavy metals in a variety of substrates and soils, bioaugmentation, and the development of bioreactors to remove heavy metals from polluted effluents and soils [6, 11]. This bacterium has just been detected for the first time as an invasive human organism [8] and has been discovered in lung sputum of cystic fibrosis sufferers [4] similar to the closely related bacterium *Ralstonia pickettii* [15].

Detection of this organism is important, as it has potential to contaminate industrial processes and as a clinically important pathogen. No biochemical test kit that can identify these bacteria is available [9], and while PCR primers exist to identify *C. respiraculi* [4], *Ralstonia* and *Cupriavidus* species [4] and closely related *Ralstonia* species *R. pickettii*, *R. mannitolilytica* [5] and *R. insidiosa* [3], there is no PCR to identify *C. metallidurans*. Identification must be carried using either classical biochemical testing or 16S ribosomal RNA (rRNA) gene sequencing, both of which can be time consuming. PCR primers for quick identification of *C. metallidurans* are presented.

Materials and methods

Bacteria used in this study

Primers were tested against three *C. metallidurans* strains LMG1195 (type strain CH34, wastewater, Belgium), CCUG43015 (human cerebrospinal fluid, Sweden) and CCUG45957 (pharmaceutical industry, Sweden). Primers were also tested against at least one strain each of *C. eutrophus*, *C. basilensis*, *C. campinensis*, *C. gilardii*, *C. oxalaticus*, *C. pauculus*, *C. respiraculi* and *C. taiwanensis* and all five *Ralstonia* species and at least one strain

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Table 1 List of strains used in this study

Species	Strain
Cupriavidus metallidurans	LMG1195, CCUG45957, CCUG43015
Cupriavidus basilensis	LMG18990
Cupriavidus campinensis	LMG19282
Cupriavidus eutrophus	LMG1199
Cupriavidus gilardii	LMG5886
Cupriavidus oxalaticus	LMG2235
Cupriavidus pauculus	LMG3244
Cupriavidus respiraculi	LMG21510
Cupriavidus taiwanensis	LMG19424
Ralstonia insidiosa	LMG21421, ATCC42129
Ralstonia mannitolilytica	LMG6866
Ralstonia pickettii	JCM5969, CCUG18841, ULC193, ULI188, ULC297, ULI171, ULI193
Ralstonia solanacearum	20S
Ralstonia syzygii	DSM7385
Campylobacter jejuni	UL-CV48
Chromobacterium violaceum	CV026
Agrobacterium tumifaciens	NTI
Escherichia coli	ATCC25922, NCTC50192
Pseudomonas aeruginosa	ATTC27853, CIP104116, PA01
Pseudomonas fluorescens	DSM50090
Salmonella enterica serovar Typhimurium	CL1
Bacillus cereus	DSM31
Bacillus thuringiensis	Var kurstaki
Enterococcus faecalis	ATCC29212
Staphylococcus epidermidis	NCTC11407
Staphylococcus aureus	ATCC25923, ATCC29213

each of a wide variety of Gram-negative and Gram-positive bacteria. The full list of strains is presented in Table 1. All *Ralstonia*/*Cupriavidus* strains were stored at −20°C in nutrient broth (NB) with 50% glycerol. All others were stored at −20°C in NB with 20% glycerol.

PCR primer design

A unique sequence was searched for in the genome of *C. metallidurans* CH34 (accession numbers NC_007973 for Chr 1 and NC_007974 for Chr 2) by comparison with other *Cupriavidus* and *Ralstonia* sp. genomes. The signal transduction histidine kinase (*hisKA*-Rmet_4605) was identified as a potential target for species-specific PCR. The HisKA protein has a PAS domain. PAS domains are internal sensors of oxygen, redox potential and light in organisms. These PAS domains play a role in regulation of circadian rhythms, activation of xenobiotic response and cell fate determination, respectively [12, 16].

Based on the HisKA nucleotide sequence of *C. metallidurans* strain CH34 available in the National Center for Biotechnology Information (NCBI) database, a PCR primer pair was designed using Primer3 software (version 0.4.0) [14]. The forward primer Cm-F1 AGTTTCCTGGC CATGATGAG and the reverse primer Cm-R1 TCCGT TTCCTGTACCACCTC were selected for further study.

In silico PCR

Amplification using the Cm-F1/Cm-R1 primers against 35 sequenced genomes, 9 total sequenced *Ralstonia* and *Cupriavidus* strains (*Cupriavidus eutrophus* H16 and *C. pinatubonensis* JMP134, *C. taiwanensis* LMG19424, *Ralstonia solanacearum* CFBP2957, PSI07 and GMI1000, *R. pickettii* 12J and 12D and *C. metallidurans* CH34 itself) and 26 total sequenced genomes of the closely related *Burkholderia* genera, were analysed using the *in silico* PCR software program available online at: http://insilico.ehu.es/PCR/ [2]. This *in silico* PCR software integrates complete genome sequencing only and allows for only two mismatches between primers and template, making the stringency of the PCR high.

PCR conditions

PCR assays were performed in 25 µl reaction mixtures, containing 5 µl DNA solution, 1 U *Taq* polymerase (Bioline, London, UK), 200 µM deoxynucleotide triphosphate (Bioline), 5 mM MgCl2 (Bioline), 1× PCR buffer (Bioline) and 25 pmol of each oligonucleotide primer (MWG Biotech, Ebersberg, Germany). DNA was prepared by boiling a picked colony at 95°C for 10 min. Lysate (5 µl) was used as a PCR template after being vortexed and centrifuged.

Amplification was carried out with a GeneAmp 2400 thermocycler (PerkinElmer Applied Biosystem). After an initial denaturation step of 5 min at 95°C, 35 amplification cycles were applied, consisting of 30 s denaturation at 95°C, 1 min primer annealing at 59°C for the first 10 cycles, followed by primer annealing at 54°C for the next 25 cycles, and a 1 min primer extension at 72°C, followed by a final extension step of 72°C for 9 min and cooling to 4°C. The PCR reaction mixture was analysed using 1% (w/v) agarose gels in 0.5× Tris–borate–ethylenediamine tetraacetic acid (TBE) buffer with 10 mg/ml ethidium bromide, using a molecular weight marker (100 bp DNA ladder, Bioline). *C. metallidurans* CH34 was used as positive control.

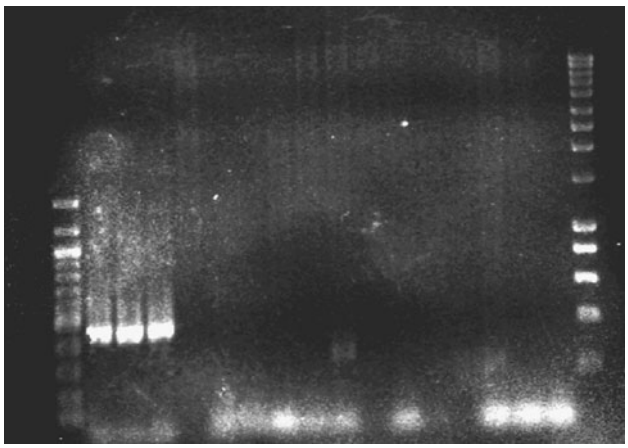

Fig. 1 Agarose gel of *HisKA* PCR products amplified from *Ralstonia* spp. *Lanes*: *1* molecular weight marker (100 bp DNA ladder, Biolab); *2 C. metallidurans* LMG 1195; *3 C. metallidurans* CCUG 45957; *4 C. metallidurans* CCUG 43015; *5 R. campinensis* LMG 19282; *6 C. basilensis* LMG 18990; *7 C. necator* LMG 1199; *8 R. pickettii* DSM 6297; *9 C. oxalaticus* LMG 2235; *10 C. pauculus* LMG 3244; *11 C. gilardii* LMG 5886; *12 R.. mannitolilytica* LMG 6866; *13 C. taiwanensis* LMG 19424; *14 R. solanacearum* AW1-A18; *15 R. insidiosa* LMG 21421; *16 C. respiraculi* LMG 21510; *17 R. syzygii* DSM7385; *18* molecular weight marker (10000 bp DNA ladder, Biolab)

Results and discussion

The in silico results with primer set Cm-F1/Cm-R1 gave a 437-bp PCR product only for *C. metallidurans* CH34, all other strains being negative. The primer set was also analysed using the BLASTn tool [1] and was found to be specific for only *C. metallidurans* CH34.

In the in vitro testing of the Cm-F1/Cm-R1 primers, only the *C. metallidurans* strains showed the 437-bp amplicon (Fig. 1). The identity of the PCR products was confirmed by sequencing (FR775896–FR775898). The results showed 100% (LMG1195), 97.7% (CCUG43015) and 97.5% (CCUG45957) identity to the published *hisKA* sequences from *C. metallidurans* CH34 (Rmet_4605). The *hisKA* primer set gave negative results for the 36 other strains that were tested, including nine species within the *Cupriavidus* genus and the five species within the *Ralstonia* genus. The PCR primer based on the *hisKA* gene in *C. metallidurans* offers a specific detection system for *C. metallidurans* strains. The PCR primers do not detect *hisKA* genes in any other of the closely related 148 strains, and the gene is also not detected in a selection of other nonrelated Gram-positive and Gram-negative strains. We offer the *hisKA* PCR primers Cm-F1/Cm-R1 designed in this study as a discriminatory selection tool for identifying *C. metallidurans* in the environment and as a useful supplement to distinguish *C. metallidurans* from other species of the genus *Cupriavidus* and the closely related *Ralstonia* genus.

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