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ScienceDirect
Journal of Microbiological Methods 70 (2007) 209–213
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Note

PCR amplification of hydrogen cyanide biosynthetic locus *hcnAB* in *Pseudomonas* spp.

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Received 8 December 2006; received in revised form 27 March 2007; accepted 27 March 2007
Available online 6 April 2007

Abstract

A PCR-based assay targeting *hcnAB*, essential genes for hydrogen cyanide (HCN) biosynthesis, allowed sensitive detection of HCN^+ pseudomonads between logs 2.9 and 3.5 cells per PCR reaction tube. RFLP analysis revealed 13 allele combinations among selected 2,4-diacetylphloroglucinol-producing (Phl^+)HCN^+, and 13 alleles in Phl^- HCN^+ strains from a global collection.

Keywords: *Pseudomonas*; Hydrogen cyanide (HCN); Diversity

Production of hydrogen cyanide (HCN), as well as production of 2,4-diacetylphloroglucinol (Phl), is an important biocontrol determinant (Haas and Defago, 2005). In *Pseudomonas fluorescens* strains (e.g., Q2-87, CHA0), the *hcnABC* genes encode for HCN synthetase critical for HCN production (Haas and Defago, 2005). Very little is known about the polymorphism of these genes. The phylogenies based on partial *hcnBC* sequences determined from a worldwide collection of Phl^+ HCN^+ biocontrol fluorescent *Pseudomonas* spp. and the deduced protein sequences revealed four main bacterial groups (Ramette et al., 2003). Because the specificity and sensitivity of previously established *hcnBC* primers have proven insufficient for analysis of mixed pseudomonad populations from grapevine (Svercel unpublished) and to detect HCN^+ but Phl^- pseudomonads, *hcnAB* specific primers were developed and validated. Additionally, *hcnAB*-RFLP analysis allowed us to study the variety in our pseudomonad collection and present a basic overview about *hcnAB* allelic diversity in HCN^+ (Phl^+ and Phl^-) strains.

Primers for *hcnAB* genes were designed with MultAlin (Corpet, 1988) from the consensus of the *hcn* sequences between *P. fluorescens* strain CHA0 (accession number AF053760) and *P. aeruginosa* strain PAO1 (AF208523). Regions of the alignment were scanned for areas with high sequence identity that could be used as priming sites for PCR amplification. Potential priming sites were selected based on the following criteria for the annealing primer: (i) ≥90% identity of primer to compared sequences, (ii) a Tm ≥55 °C, (iii) priming site ≥350 bp distant from that of nearest complementary primer and (iv) a C or G in the terminal 3′ position. Optimal amplification and specificity were obtained using forward primer PM2 (31-mer 5′-TGCGGCATGGGCGTGTGCCATTGCTGCCTGG-3′) and reverse primer PM7-26R (26-mer 5′-CCGCTCTTGATCTGCAATTGCAGGCC-3′) (synthesized by MWG Biotech, Basel, Switzerland). Amplifications were carried out in 12-μl reaction mixtures containing 4 μl of lysed bacterial suspension, 1× PCR buffer (Amersham Pharmacia, Uppsala, Sweden), bovine serum albumin (0.5 g l^{-1}; Fluka, Buchs, SG, Switzerland), 5% dimethyl sulfoxide (Fluka), 100 μM each of dATP, dCTP, dGTP and dTTP (Amersham Pharmacia), 0.40 μM of each primer and 1.4 U of *Taq* DNA polymerase (Amersham Pharmacia). The PCR started with the initial denaturation (2 min at 94 °C) was followed by 35 cycles of 94 °C for 30 s, 67 °C for 30 s and 72 °C for 60 s and final

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doi:10.1016/j.mimet.2007.03.018

extension at 72 °C for 10 min. Amplifications were performed with a PTC-100TM cycler (MJ Research Inc., Watertown, MA), and the resulting PCR products were separated in 1.5% agarose gels in 0.5x Tris–borate–EDTA (TBE) buffer at 160 V for 1 h. The amplified fragment was 570 bp and included 136 bp of *hcnA* (312 nucleotides) and 434 bp of *hcnB* (1404 nucleotides) (Fig. 1).

A total of 57 Phl^+ HCN^+ strains representing each of the four previously described phylogenetic HCN groups of biocontrol pseudomonads (Ramette et al., 2003) were used to test the specificity of primers (Table 1). Additionally, the specificity of primers was tested on 41 diverse Phl^- HCN^+ strains (Table 1). *P. fluorescens* strains 2–79 and P3 were included as HCN-negative controls. All pseudomonads were routinely grown at 27 °C on King's B agar (KBA, King et al., 1954) and stored at −80 °C in 40% glycerol. DNA preparation was done as described by Wang et al. (2001).

To test the specificity of the primers, all strains were adjusted to a constant concentration of approximately 10^9 cells per ml. To test the sensitivity of the assay for detection of $hcnAB^+$ strains in a background of $hcnAB^-$ bacteria, two different types of template mixtures were prepared. The first mixture consisted of a ten-fold serial dilution of strain CHA0 or Q2-87 (1×10^9 to 1×10^3 cells per ml) in a constant concentration of negative control strain P3 (1×10^9 cells per ml). The second mixture consisted of ten-fold serial dilution of a 24:1 mixture of strain P3 with either strain CHA0 or Q2-87 (1×10^8 to 1×10^3 cells per ml). Dilutions were initially frozen at −80 °C for a minimum of 1 h, and then transferred to a −20 °C freezer for storage.

A single amplicon of about 570 bp in length was obtained for all HCN^+ strains using our PCR method, whereas no amplicon was obtained from the two negative HCN pseudomonads. When we varied the concentration of HCN^+ bacteria in samples but kept a fixed background population of HCN^- bacteria (e.g., P3 at log 7.5 cells per PCR reaction tube) we were able to detect HCN^+ strains present at between log 2.9 cells (CHA0) and log 3.2 cells (Q2-87) per PCR reaction tube. When we tested dilutions of mixtures containing a fixed proportion of high background (i.e., 24:1 of P3:CHA0 or P3:Q2-87), a clear amplification signal was obtained with an average of log 3.1 $hcnAB^+$ per PCR tube of CHA0 and log 3.5 per PCR tube of Q2-87. This demonstrates that HCN^+ bacteria can be sensitively detected in samples where they represent a low percentage of the total pseudomonad community and/or where the numbers are low regardless of their relative proportion to the total community, which is an important feature for environmental biodiversity analyses.

To characterize $hcnAB^+$ alleles, 5 μl of amplified product were used for restriction analysis with 1.5 U of *Hae*III, *Msp*I or *Taq*I enzymes (Boehringer, Mannheim). The combination of just these three digests was sufficient to discriminate all *phlD* alleles and the achieved polymorphism corresponded exactly to that defined previously by BOX-PCR genomic fingerprinting in Phl^+ pseudomonads (McSpadden Gardener et al., 2001). Reactions

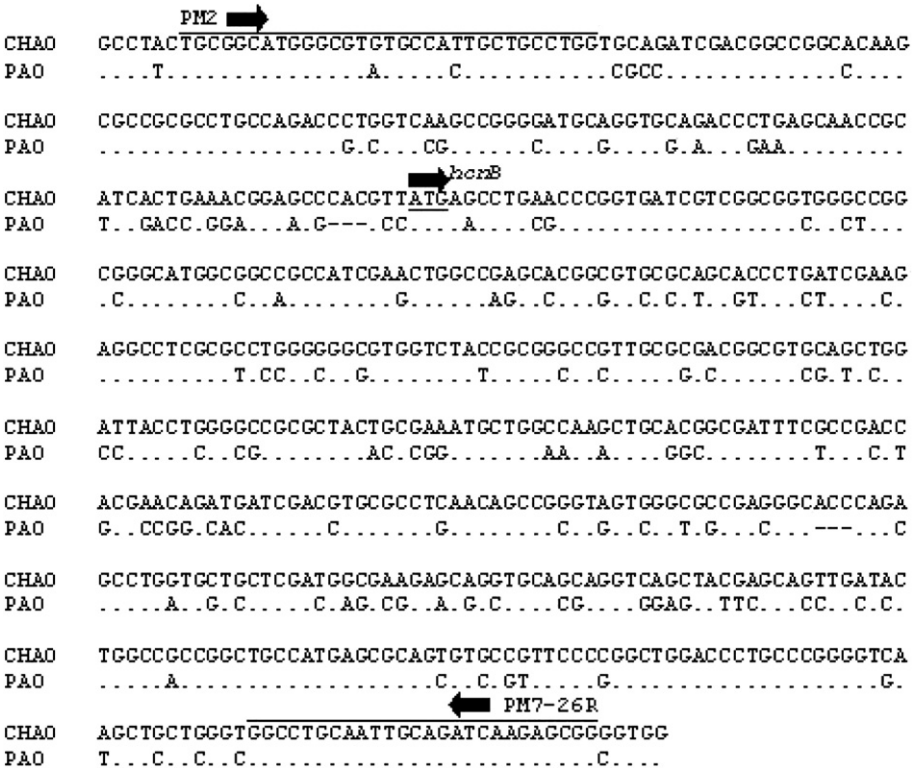

Fig. 1. Alignment of partial *hcnAB* sequences of *Pseudomonas fluorescens* CHA0 (accession number AF053760) and *P. aeruginosa* PAO (AF208523). Dots and dashes represent conserved bases and alignment gaps, respectively. The *hcnB* start codon is underlined and the sites annealing to the PCR primers PM2 and PM7-26R are overlined.