Cloning and analysis of agarase-encoding gene from *Paenibacillus* sp.

R. Meškienė,
V. Časaitė,
L. Marcinkevičienė,
I. Bachmatova,
R. Meškys*

Department of Molecular Microbiology and Biotechnology,
Institute of Biochemistry,
Mokslininkų 12,
Vilnius LT-2600, Lithuania

Agar is a family of related polymers sharing a common backbone of neoagarobiose bound through β-1,4 bonds. Several new bacterial strains capable of producing extracellular agarases were isolated from soil. Based on 16S rRNA gene sequencing and RFLP analysis, the isolates H9 and H1 were found to belong to the genus *Paenibacillus* and *Flexibacter-Flavobacter* group, respectively. The agarase-encoding gene was cloned from the *Paenibacillus* H9 strain on its ability to secrete an active enzyme in *Escherichia coli* cells. Partial sequencing of the cloned gene showed that agarase from *Paenibacillus* sp. H9 is most similar to beta-agarases A and B from *Vibrio* sp. strain JT0107 (27% and 31% identity, respectively, according to the Fasta analysis).

Key words: *Paenibacillus*, agarase-encoding gene, 16S rRNA

INTRODUCTION

Agar is one of the most thoroughly characterized polysaccharides, which occurs in cell walls of some red algae and is composed of agarose and agaropectin [1]. Hydrolytic enzymes that degrade agarose are classified into two groups: α- and β-agarases. Agarolytic enzymes have been purified from various microorganisms, including *Pseudomonas* [2], *Cytophaga* [3], *Streptomyces* [4], *Vibrio* [5], *Alteromonas* [6], *Pseudoalteromonas* [7] and *Alterococcus* [8]. The agarase encoding genes have been cloned from various microorganisms assigned to the genera *Streptomyces* [9–11], *Pseudomonas* [12, 13], *Vibrio* [14, 15]. Most of previously described agarolytic microorganisms have been isolated from marine or related environment. The aim of the current study is to describe the isolation of new soil-borne bacterial strains producing agarases and cloning the agarase-encoding gene from one isolate.

MATERIALS AND METHODS

Chemicals. Yeast extract, X-Gal, isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma. Tryptose was from Serva. Restriction endonucleases and T4 DNA ligase were obtained from MBI (Vilnius, Lithuania) and were used according to the manufacturer's instruction.

Bacterial strains. *Escherichia coli* DH5α [ϕ80*dlacZΔM15* Δ(*lacZY-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1*] was used as the recipient strain for all cloning experiments. The agarase-producing microorganisms were isolated throughout this work.

Media. TY broth and agar [16] were used as complete media. Ampicillin (50 μg/ml) was added to the medium when required to select for plasmids. All bacterial strains were grown at 30 °C.

Enrichment cultures and isolation. The soil samples were suspended in sterile saline and diluted. A volume of each dilution was spread on TY agar and the plates were incubated at 37 °C for 3–5 days. Colonies that caused agar liquefaction were picked off and purified by successive streaking on TY agar plates.

Determination of agarase activity. The formation of reducing sugars was determined with the 3,5-dinitrosalicylic acid reagent according to the previously described procedures [8]. In short, cultures were incubated in the presence of 0.1% agarose at 37 °C for an appropriate time, centrifuged to remove bacterial cells and gel residues. 1 ml of culture supernatant was mixed with 3 ml of 3,5-dinitrosalicylic acid reagent, heated at 100 °C for 5 min, cooled, and diluted with 20 ml of water. Optical density was read at 500 nm and values for reducing sugars were expressed as galactose.

Characterisation of isolates. Standard microbiological tests and analyses were performed. 16S rRNA encoding genes (1.5 kb fragments) were amplified using the universal primers W001 (5'-AGAGTTTGATCM

*Corresponding author.
E-mail address: rmeskys@bchi.lt

TGGCTC-3') and W002 (5'-G N T A C C T T G T T A C G ACTT-3') [17]. The PCR products were purified with a DNA purification kit and cloned into pTZ57R/T plasmid (Fermentas MBI, Lithuania). Each cloned 16S ribosomal DNA was sequenced in both orientations by using the ABI377 system.

Preparation, analysis, and cloning of DNA. Total DNA was isolated from H1 and H9 strains by the method of Woo et al. [18]. Plasmid DNA was isolated from *E. coli* as described [19]. Competent cells of *E. coli* were prepared as described [20].

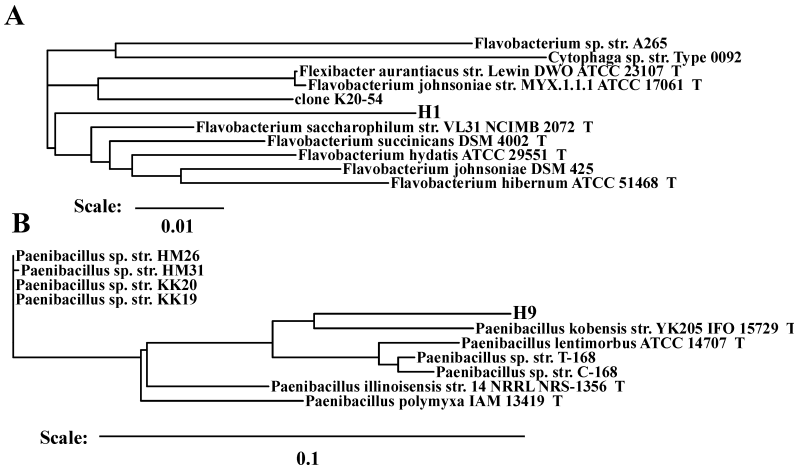

Fig. 1. Unrooted tree showing the phylogenetic relationships of strain H1 within the *Flexibacter-Flavobacter* group and strain H9 within the genus *Paenibacillus* (B). The trees were constructed using the neighbour-joining method (The Ribosomal Database Project (RDPII), http://rdp.cme.msu.edu/html/ [23]) based on a comparison of approximately 1000 nucleotides

RESULTS AND DISCUSSION

Strain isolation and characterisation. Several soil-born bacterial strains forming depressions or pits on the agar surface were isolated. Two of these isolates, H1 and H9, exhibiting the highest agarase activity both on plates and in a liquid medium were selected for further studies. The H1 and H9 strains were found to be gram-variable long and gram-positive short rods, respectively, both capable of using aerobically both peptone and ammonium as a nitrogen source and grow on simple defined media consuming agar as a sole carbon and energy source. The 16S rRNA encoding genes were amplified from both bacteria, and the RFLP analysis was carried out. The results of this analysis demonstrated differences between the H1 and H9 strains and known agarolytic microorganisms. The sequences (approximately 1500 bp) of the 16S rRNA encoding genes from H1 and H9 strains showed a strong homology to 16S rDNA from the *Flexibacter-Flavobacter* group (Fig. 1, A) and *Paenibacillus* spp. (Fig. 1, B), respectively. Since any agarase has not been previously described from the latter organism and only one β-agarase from the related marine bacterium *Bacillus cereus* ASK202 has been purified [21], *Paenibacillus* sp. H9 was chosen for further gene cloning work.

Cloning of agarase encoding gene. Total DNA from *Paenibacillus* sp. H9 was partially digested with *Pst*I and ligated into the *Pst*I-digested plasmid cloning vector pTZ18R. *E. coli* DH5α was transformed with a ligation mixture and the transformants were selected on TY agar plates containing ampicillin, IPTG and X-Gal. Bacteria carrying the cloned agarase gene formed white colonies and produced pits on the agar surface. In this way a depression-forming colony was identified, which carried a 6.5 kb *Pst*I–*Pst*I fragment inserted into pTZ18R. A restriction enzyme cleavage map of this recombinant plasmid (pAH43) was then generated (Fig. 2). The restriction map of the cloned region differed essentially from all previously published maps [7–14, 22]. From these results we concluded that a novel agarase-encoding gene was isolated. Deletion analysis and partial sequencing of the pAH43 plasmid showed that two different genes encoding agarases are localized one after the other in the cloned DNA fragment from *Paenibacillus* sp. strain H9 (Fig. 2). It has been previously known that only *Vibrio* sp. JT0107 among all described agarolytic microorganisms produces two agarases whose genes are localized in one operon [15]. It is worth noting that agarases from the *Paenibacillus* sp. H9 are most similar to β-agarases A and B from *Vibrio* sp. strain JT0107 (27% and 31% identity, respectively), too.

Additional work will be required to elucidate the properties and differences of both agarases from *Pae-*

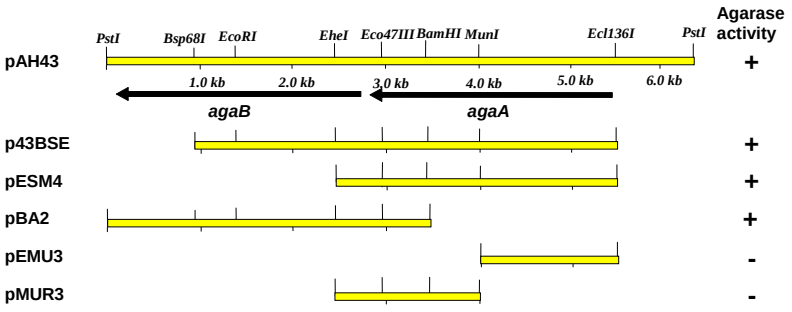

Fig. 2. Restriction map and deletion analysis of the cloned DNA fragment containing agarase-encoding genes from *Paenibacillus* sp. H9.

nibacillus sp. H9. The protein purification is in progress. Finally, the cloned genes could be exploited to overproduce the agarase enzymes which could be used to dissect the chemical structure of complex polysaccharides such as agar or possibly to produce commercially useful products.

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PAENIBACILLUS SP. AGARAZĖS GENO KLONAVIMAS IR ANALIZĖ

S a n t r a u k a

Iš dirvožemio buvo išskirti agarazę produkuojantys bakteriniai kamienai. Remiantis 16S rRNR genų sekomis ir jų restrikcinių fragmentų ilgio polimorfizmo tyrimais, nustatyta, kad kamienas H1 priklauso *Flexibacter-Flavobacter* bakterijų grupei, o kamienas H9 yra *Paenibacillus* genties atstovas. Iš pastarojo organizmo buvo klonuotas DNR fragmentas, koduojantis aktyvią agarazę *Escherichia coli* ląstelėse. Nustatyta, kad *Paenibacillus* sp. H9 koduoja dvi agarazes, o jų sekos yra panašios į *Vibrio* sp. JT0107 produkuojamų agarazių sekas.