Microbiological Research 186–187 (2016) 52–61

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Discovering novel enzymes by functional screening of plurigenomic libraries from alga-associated *Flavobacteriia* and *Gammaproteobacteria*

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ARTICLE INFO

Article history:
Received 4 February 2016
Received in revised form 17 March 2016
Accepted 22 March 2016
Available online 24 March 2016

Keywords:
Alga-associated microflora
Marine bacteria
Agarase
Iota-carrageenase
Kappa-carrageenase
Esterase
Beta-glucosidase
Xylanase
Marine enzymes

ABSTRACT

Alga-associated microorganisms, in the context of their numerous interactions with the host and the complexity of the marine environment, are known to produce diverse hydrolytic enzymes with original biochemistry. We recently isolated several macroalgal-polysaccharide-degrading bacteria from the surface of the brown alga *Ascophyllum nodosum*. These active isolates belong to two classes: the *Flavobacteriia* and the *Gammaproteobacteria*. In the present study, we constructed two "plurigenomic" (with multiple bacterial genomes) libraries with the 5 most interesting isolates (regarding their phylogeny and their enzymatic activities) of each class (*Fv* and *Gm* libraries). Both libraries were screened for diverse hydrolytic activities. Five activities, out of the 48 previously identified in the natural polysaccharolytic isolates, were recovered by functional screening: a xylanase (GmXyl7), a beta-glucosidase (GmBg1), an esterase (GmEst7) and two iota-carrageenases (Fvi2.5 and Gmi1.3). We discuss here the potential role of the used host-cell, the average DNA insert-sizes and the used restriction enzymes on the divergent screening yields obtained for both libraries and get deeper inside the "great screen anomaly". Interestingly, the discovered esterase probably stands for a novel family of homoserine o-acetyltransferase-like-esterases, while the two iota-carrageenases represent new members of the poorly known GH82 family (containing only 19 proteins since its description in 2000). These original results demonstrate the efficiency of our uncommon "plurigenomic" library approach and the underexplored potential of alga-associated cultivable microbiota for the identification of novel and algal-specific enzymes.

1. Introduction

For a long time, researchers have investigated environmental samples to identify novel microbial biocatalysts. Early efforts focused on discovering novel enzymes of known or newly described cultivable microorganisms. More recently, (sequence- or function-based) metagenomic screening has been included among the tools used to mine for novel microbial enzymes (Handelsman, 2004). Sequence-based metagenomic approaches provide access to non-cultivable microorganisms, but as enzymes are sought by sequence comparisons with known biocatalysts, only new variants of existing enzymes are discovered (Simon and Daniel, 2011). In contrast, function-based screening of metagenomic libraries can lead to discovering completely novel enzymes, while looking only at the function of the gene and not its similarities with known sequences. Yet searching for a particular enzyme type in a metagenome, is somewhat like looking for a needle in a haystack. It is fastidious, and yields are poor (Ekkers et al., 2012; Ferrer et al., 2016).Therefore the old-fashioned culturing approach, followed by selection of isolates showing activities of interest, still has a place in the hunt for novel enzymes. Preselection of "active" strains limits the number of genomes to be prospected and allows more focused work. Furthermore, even though culturing methods restrict the diversity of the findings, as only a very low percentage of existing microbes are cultivable (estimated at less than 1% in most environmental samples (Pace, 1997; Torsvik and Øvreås, 2002)), novel enzymes and enzyme families far different from known ones can still be discovered in underexplored bacterial taxa and environments.

Bacteria associated with algae have been shown to produce many polysaccharide hydrolases, because of their complex and

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http://dx.doi.org/10.1016/j.micres.2016.03.005
0944-5013/

dynamic interactions with their hosts (Holmström et al., 2002; Martin et al., 2014b). We have recently demonstrated that the cultivable microbiota associated with the brown alga *Ascophyllum nodosum* is rich in macroalgal-polysaccharide-degrading bacteria (Martin et al., 2015). Furthermore, many novel species and genera have been identified in alga-associated microbiotas, suggesting that they constitute an interesting biotic environment for the discovery of new bacterial taxa and hence, original biocatalysts (Goecke et al., 2013). In the class *Flavobacteriia* (*Bacteroidetes*) several polysaccharolytic genera were first identified on a macroalga: for example, *Cellulophaga* on the brown alga *Fucus serratus*, *Formosa* on the brown alga *Fucus evanescens*, and *Zobellia* on the red alga *Delesseria sanguine* (Barbeyron et al., 1989; Ivanova et al., 2004; Johansen et al., 1999). Novel glycoside hydrolase families have also been discovered in these genera (Michel and Czjzek, 2013), such as the iota-carrageenase (GH82) and β-porphyranase (GH86) families in *Zobellia galactanivorans* (Barbeyron et al., 2000; Hehemann et al., 2010). On the other hand, few studies have focused on identifying more classical hydrolytic enzymes in alga-associated bacterial species, such as cellulases, xylanases, beta-glucosidases, or esterases, even though marine hydrolases differ markedly from their terrestrial homologs (Dalmaso et al., 2015). It thus seems that cultivable alga-associated bacteria constitute an interesting source of a large range of novel hydrolytic enzymes.

In a previous study, we isolated and identified several algal-polysaccharide-degrading bacteria from the microflora associated with the brown alga *A. nodosum* (Martin et al., 2015). All the isolates, some of which very probably represent new species, were taxonomically assigned to the classes *Flavobacteriia* and *Gammaproteobacteria*. They display diverse hydrolytic activities. In the present work, to identify novel enzyme-encoding genes from these original alga-associated bacteria, we have used an uncommon approach: constructing and screening "plurigenomic" (multiple bacterial genomes) libraries. One library was constructed with the genomes of five *Flavobacteriia* isolates and one with the genomes of five *Gammaproteobacteria* isolates. Screening was done for agarase, iota-carrageenase and kappa-carrageenase, beta-glucosidase, endo-cellulase, xylanase, and esterase activity.

2. Materials and methods

2.1. Isolation and functional screening of alga-associated Flavobacteriia *and* Gammaproteobacteria

Diverse algal-polysaccharide-degrading bacteria were isolated from the brown alga *A. nodosum*, as described in our previous study (Martin et al., 2015). These bacteria were assigned to the classes *Flavobacteriia* and *Gammaproteobacteria*. The isolates were tested for diverse hydrolytic activities at room temperature. Agarase, iota-carrageenase and kappa-carrageenase activities were detected on Marine Broth (Difco) containing, respectively, 1.5% agar, 2% iota-carrageenan, or 1% kappa-carrageenan (Sigma). Isolates showing hydrolytic activity were detected by a hole in the surrounding jellified medium for agarase and κ-carrageenase activities or the complete liquefaction of the medium for ι-carrageenase activity. Marine Agar (Difco) and AZCL-HE-cellulose, −amylose or −xylan (birchwood) (Megazyme) was used to detect endo-cellulase, alpha-amylase, or *endo*-1,4-β-D-xylanase activity, respectively. A blue halo around a colony was indicative of hydrolase activity. Lipolytic activity was detected on Marine Agar and 3% Difco Lipase Reagent (Difco). Lipolytic isolates were detected by a clear halo around the colony. Beta-glucosidase activity was detected as described by Mattéotti et al. (2011) on Marine Agar containing 0.5% esculin and 0.1% ammonium iron (III) citrate (Sigma-Aldrich). A bacterium was identified as positive when a brown precipitate appeared around its colony.

2.2. Construction of plurigenomic libraries with Flavobacteriia and Gammaproteobacteria isolates

Two plurigenomic libraries were constructed, one for each class. Five isolates per class were used. The *Flavobacteriia* used were the *Cellulophaga* isolates An8, An9, and An20 (16s rRNA sequence accession numbers: LN881186, LN881202, LN881252), the *Zobellia* isolate An14 (LN881227), and the *Maribacter* isolate An21 (LN881276). The *Gammaproteobacteria* used were the *Shewanella* isolates An4 and An36 (LN881152, LN881379), the *Pseudoalteromonas* isolate An33 (LN881360), the *Colwellia* isolate An23 (LN881284), and the *Paraglaciecola* isolate An27 (L881305). These ten isolates were selected on the basis of their original phylogeny and the intensity and/or diversity of their activities (Table 1). They were grown for 24–48 h at 20 °C in 3 ml Zobell medium (Difco Marine Broth). Genomic DNA from each isolate was extracted as described by Cheng and Jiang (2006). DNA quantity and quality were checked, respectively, with the Qubit fluorometer (Invitrogen) and by gel electrophoresis through a 0.8% agarose gel. The genomic DNA of each strain was tested for restriction by *Sau3AI* or its isoschizomer *DpnII*. We then pooled, on the one hand, 3 μg genomic DNA of each *Flavobacteriia* isolate, and on the other hand, 3 μg genomic DNA of each *Gammaproteobacteria* isolate. The pool of genomic DNA from the *Flavobacteriia* isolates was partially restricted for 1–3 min with 0.2 U/μg DpnII (NEB), and the *Gammaproteobacteria* pool for 1–2 min with 0.3 U/μg *Sau3AI* (Roche). After elimination of small DNA inserts by size-selective polyethylene glycol-NaCl precipitation as described by Biver and Vandenbol (2013), the DNA was purified by migration through a 1% low-melting-point agarose gel (Promega). DNA inserts exceeding 5 kb were recovered by beta-agarase digestion (NEB). The cloning vector *pHT01* (MoBiTec, Germany) was linearized with *BamHI* (Roche) and dephosphorylated (Dephos and Ligation kit, Roche). A vector:insert ratio of 1:3 was used to ligate the DNA inserts of each library into the *pHT01* cloning vector at 16 °C overnight (T4 DNA ligase, Roche). Electrocompetent Electromax™ *Escherichia coli* DH10B cells (Life Technologies) were transformed with 1 μl ligation products. The average DNA insert size in each plurigenomic library was estimated by isolation and purification of 20 randomly chosen plasmids.

2.3. Functional screening of the plurigenomic libraries

Almost 12600 clones of the *Gammaproteobacteria* (Gm) library and 15000 clones of the *Flavobacteriia* (Fv) library were isolated in 96-well plates and grown overnight in 2xYT liquid culture medium at 37 °C. The liquid cultures were then replicated onto the various screening media and incubated at room temperature. Similar screening tests were used as described above, but Marine Broth was replace by minimal medium (1 g/l Yeast Extract, 5 g/l Bactotryptone (MP, Biomedicals), 5 g/l NaCl (Merk)) and Marine Agar by minimum medium with 1.5% agar. The *Fv* library was screened for agarase, iota- and kappa-carrageenase, esterase, cellulase and xylanase activities and the *Gm* library was additionally screened for beta-glucosidase activity, as regards the hydrolytic activities observed for the natural strains of both libraries (Table 1). Positive clones were isolated and their plasmids purified. Activity was confirmed by transforming other *E. coli* strains (DH5α) with the purified plasmid and testing recombinant clones on the screening medium corresponding to the observed activity.