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Construction and screening of a functional metagenomic library to identify novel enzymes produced by Antarctic bacteria

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Abstract A metagenomic fosmid library of approximately 52 000 clones was constructed to identify functional genes encoding cold-adapted enzymes. Metagenomic DNA was extracted from a sample of glacial meltwater, collected on the Antarctic Peninsula during the ANTARKOS XXIX Expedition during the austral summer of 2012–2013. Each clone contained an insert of about 35–40 kb, so the library represented almost 2 Gb of genetic information from metagenomic DNA. Activity-driven screening was used to detect the cold-adapted functions expressed by the library. Fifty lipase/esterase and two cellulase-producing clones were isolated, and two clones able to grow on Avicel® as the sole carbon source. Interestingly, three clones formed a brown precipitate in the presence of manganese (II). Accumulation of manganese oxides was determined with a leucoberbelin blue assay, indicating that these three clones had manganese-oxidizing activity. To the best of our knowledge, this is the first report of a manganese oxidase activity detected with a functional metagenomic strategy.

Keywords **metagenomics, manganese oxidase, esterase, functional screening, cellulase, Antarctica**

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1 Introduction

Antarctic microorganisms must deal with desiccation, high UV radiation, and cold. The adaptation of their enzymes to function properly at low and even freezing temperatures is one of the main mechanisms allowing microorganisms to survive in this cold environment[1]. Together with other adaptations, this has allowed bacteria to colonize almost every corner of the Antarctic continent[2].

Different strategies have been used to study and characterize the bacterial communities in Antarctic, most based on bacterial isolation in growth media. However, it is estimated that only around one in every 100 microbes can grow and divide under standard laboratory conditions, a fact known as the "great plate count anomaly"[3]. Diverse approaches have been developed to improve our understanding of the missing biodiversity among the 99% of microbes considered "unculturable". One of the most promising methodologies is metagenomics, also referred to as "environmental genomics" or "community genomics". Whole-metagenome sequencing approaches have been used to study the community compositions, ecology, and functional capacities of bacteria in cold environments[4-6]. Functional metagenomic approaches have also been successful, allowing the functional analysis of unculturable bacteria by analyzing the heterologous expression of metagenomic DNA in a well-known bacterium, such as *Escherichia coli*[7-9]. With this approach, new cold-adapted enzymes have been identified, including proteases, lipases/esterases, amylases, and cellulases[10-13].

In this study, we constructed a metagenomic fosmid library in *E. coli* using DNA extracted from a meltwater sample collected on the Antarctic Peninsula. The library was used to screen for cold-adapted activities in selective media

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and several *E. coli* clones with lipolytic, cellulolytic, and manganese oxidase activity were successfully identified.

2 Materials and methods

2.1 Sample collection

A 50 mL sample was collected from a small pond of glacial meltwater near the coast, on the continental landmass of the Antarctic Peninsula (63°13′12.8″S, 57°13′5.4″W), during the 29th ANTARKOS Expedition organized by the Uruguayan Antarctic Institute in January 2013. Some penguins of the species *Pygoscelis adeliae* and *Pygoscelis papua* were present some meters from the pond. The pond was located in a pristine area where no anthropogenic pollution was expected because it was located far from research stations and had only difficult terrestrial access (Figure 1). Therefore, this sample was a very attractive material for a metagenomic analysis to isolate novel cold-adapted enzymes.

Figure 1 Location of the sample, showing the sampling location in relation to D'Urville Island, King George Island, and Antarctic Peninsula.

The pH of the sample was 9.0 and the temperature 1.0°C. The sample was kept on ice during transportation for about 1 h and the metagenomic DNA was immediately extracted on the ship ROU 04 General Artigas.

2.2 Metagenomic DNA isolation

The metagenomic or environmental DNA (eDNA) was isolated using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). An aliquot of the sample (300 µL) was loaded into the PowerBead Tubes provided with the kit, vortexed horizontally for 10 min, and purified according to the manufacturer's instructions. Metagenomic DNA was eluted from the silica spin filter membrane with 100 µL of 10 mM Tris solution provided with the kit. The quality and quantity of the eDNA were assessed with agarose gel electrophoresis and spectrophotometry.

2.3 Metagenomic library construction

To construct the metagenomic library, the CopyControl™ Fosmid Library Production Kit with the pCC1FOS™ fosmid vector (Epicentre Biotechnologies, Madison, WI, USA) was used. Because more than 10% of the eDNA had an average size of 30–40 kb, no further purification was performed before it was cloned. The whole eDNA was end-repaired and cloned into the pCC1FOS vector. The ligation mixture was packaged with the MaxPlax™ Lambda Packaging Extracts. Escherichia coli strain TransforMax™ EPI300™-T1^{R} (Epicentre Biotechnologies) was transformed with the packaging mixture, as recommended by the manufacturer, and the transformants were selected on solid Luria–Bertani (LB) medium[14] supplemented with 12.5 µg·mL^{-1} chloramphenicol (Cm). The library was organized in 96-well plates. LB–Cm broth (100 µL per well) was inoculated with pooled clones (~30 clones per pool). The plates were incubated at 30°C for 24 h and used for functional screening and library storage. For this purpose, inocula were transferred to fresh medium with a 48-pin replica plater and the cultures were grown at 30°C for 24 h. After the addition of 25% (v/v) glycerol, the library was stored at –80°C.

2.4 Functional screening

The metagenomic library was functionally screened by directly selecting the E. coli recombinant pools on solid LB–Cm medium supplemented with 0.01% (w/v) L-arabinose and different substrates, according to the activity to be evaluated.

All samples were incubated at 30°C for 24 h to allow *E. coli* growth, followed by further incubation at 25°C to facilitate the expression of any cold-adapted enzymes. The cultures were monitored periodically for at least 20 d to detect enzymatic activity. For assays that required reagents to be added to the bacterial cultures (e.g., to identify cellulolytic activity or peroxidases), the plates were incubated at 30°C for 24 h, followed by further incubation at 25°C for 10 d before the reagents were added.

2.4.1 Lipolytic activity

We screened for lipase/esterase-producing clones on solid medium containing 1% (v/v) emulsified tributyril (an ester of glycerol and a fatty acid with four carbons), tricaprylin (an ester of glycerol and a lipidic chain with eight carbons), or triolein (an ester of glycerol and an unsaturated fatty acid with 18 carbons) to differentiate esterases from true lipases. A clear halo around the colony was considered to indicate lipase/esterase activity[15-16].

2.4.2 Cellulose-hydrolyzing activity

Cellulolytic clones were identified on solid medium containing either 0.5% (w/v) carboxymethyl cellulose (CMC) or 0.5% (w/v) microcrystalline cellulose (Avicel® PH-101) as substrate. Cellulase activity was detected using Congo

Red staining[17]. The colonies were overlain with 0.05% (w/v) Congo Red, incubated for 10 min at room temperature, and washed with 1 M NaCl. A clear orange halo around colonies grown on CMC- or Avicel®-containing medium indicated cellulose-hydrolyzing activity.

2.4.3 Avicel® as the sole carbon source

To identify clones able to utilize cellulose as a carbon source, the cells were grown on solid M9 minimal medium supplemented with 400 μM thymine and 200 μM leucine, containing 0.5% (w/v) Avicel® as the sole carbon source. The capacity to grow on this medium identifies strains that produce cellulases and β-glucosidases, and have transport systems that internalize cellobiose or glucose.

2.4.4 Xylanase

Xylanase-producing clones were detected on plates containing 0.5% (w/v) beech wood xylan as substrate, followed by Congo Red staining[18-19]. Positive clones gave a clear orange halo around the colony.

2.4.5 Ligninolytic activity

Laccase-producing clones were screened on medium containing 2.4 mM guaiacol and 0.16 mM $CuSO_4$. Strains able to oxidize guaiacol were apparent by their production of a brownish color[20].

Peroxidase activity was assessed on medium containing 0.025% (w/v) 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate, and manganese peroxidase activity was assessed on medium containing 0.025% (w/v) ABTS plus 0.5 mM $MnCl_2$. Well-grown colonies were covered with 1.25% (v/v) H_2O_2. Peroxidase-producing bacteria were identified by the appearance of a blue color resulting from ABTS oxidation[21].

2.5 Isolation of active clones

Active bacterial pools were serially diluted and plated in selective or differential media for their direct identification, or replica plated in LB–Cm and selective media. The active clones identified were streaked onto LB–Cm medium, and the single colonies were then cultured in LB–Cm broth and stored in 25% (v/v) glycerol at –80°C.

To confirm that the genetic information of interest was contained in the cloned fosmid, it was isolated, purified, and used to transform electrocompetent E. coli strain EPI300™-T1^R. For this purpose, the active clones were grown in LB–Cm broth at 37°C until the optical density at 600 nm (OD_{600}) reached 0.5. The copy number of the fosmid was then amplified by incubating the cells with 0.01% (v/v) L-arabinose for 5 h. After incubation, the fosmid was isolated with the alkaline lysis method[14]. The size of the insert was assessed by restriction with *Eco*RI and *Hin*dIII and the prepared fosmid was used to transform electrocompetent E. coli EPI300™-T1^R cells. To obtain electrocompetent cells, an overnight culture in LB medium was diluted 200-fold and grown at 37°C until the OD_{600} reached 0.5. The cells were harvested by centrifugation at 4 000 xg rpm for 5 min at 4°C, washed with distilled water, and then with 10% (v/v) glycerol. The glycerol-washed cells were harvested at 5 000 xg for 7 min at 4°C and resuspended in 10% (v/v) glycerol. Aliquots (100 μL) were stored at –80°C. Electrocompetent *E. coli* EPI300™-T1^R cells were transformed with 1 μL of the fosmid DNA preparation with a short pulse of 1 800 V in a 0.1 cm cuvette in a MicroPulser Electroporation System (Bio-Rad, Hercules, CA, USA). Sterile distilled water and a pBlueScript SK (Stratagene) plasmid preparation were used as the negative and positive controls, respectively. The phenotypes of the transformed cells were assessed with selective and differential media.

2.6 Mn oxidation assay

The oxidation of Mn^{2+} was evaluated with the leucoberbelin blue I (LBB) assay. Colonies grown on LB–Cm medium supplemented with 0.5 mM $MnCl_2$ were overlain with a solution of 0.04% (w/v) LBB in 45 mM acetic acid. LBB oxidizes and turns blue in the presence of Mn^{+3}–Mn^{+7}[22].

3 Results

3.1 Metagenomic library construction

To avoid modification of the bacterial community, the metagenomic DNA was isolated as soon as possible, around 1 h after sampling. About 30 μg of high-quality high-molecular-weight DNA per milliliter of sample was obtained.

To identify functions that require the expression of whole operons, we decided to use the pCC1FOS™ fosmid as the vector to construct the library. This fosmid expresses a Cm-resistance activity as a selectable marker and allows DNA inserts of around 40 kb to be packaged. The use of this vector, together with *E. coli* strain EPI300™-T1^R provided with the kit, has the advantage that it allows on-demand amplification of the fosmid copy number from a single copy to ~200 copies per cell, a feature that is extremely important in functional metagenomic screening.

Using this methodology, a metagenomic library of approximately 52 000 clones was generated, containing about 2 Gb of metagenomic information (Table 1).

3.2 Functional screening of the metagenomic library

In recent years, our group has been looking for and studying enzymes suitable for the biofuel industry, specifically in the production of bioethanol from lignocellulosic biomass and biodiesel production. Therefore, we searched the library for lignocellulolytic and/or lipolytic activities. In particular, the library was screened for clones displaying cellulase (EC 3.2.1.4), xylanase (EC 3.2.1.8), laccase (EC 1.10.3.2),

Table 1 Results of metagenomic library construction and its functional screening

Metagenomic library information					Functional screening results				
Clones in the library	Size of eDNA/ clone /Kb	eDNA information of library /Gb	*E. coli* genome equivalents[a]	Clones per pool	Pools screened	Tbut[b] positive clones	CMC[c] positive clones	Avicel positive clones[d]	MnOx[e] positive clones
51 840	35	1.814	394	30	1 728	50	2	2	3

Note: a: Considering the average *E. coli* genome to be 4.6 Mb; Tbut[b]: tributyril; CMC[c]: carboxymethyl cellulose; d: Clones able to use microcrystalline cellulose (Avicel®) as the sole carbon source; MnOx[e]: manganese oxidase.

peroxidase (EC 1.11.1.14) manganese peroxidase (EC 1.11.1.13), esterase (EC 3.1.1.1), or lipase activities (EC 3.1.1.3).

We identified 50 pools with esterase/lipase activity, two pools with cellulolytic activity, and two pools capable of growing with Avicel® as the sole carbon source. No pools with xylanase, laccase, peroxidase, or manganese peroxidase activities were detected. Interestingly, we identified three pools that produced a brownish-red precipitate in the presence of manganese (Table 1).

3.2.1 Lipolytic activity

We obtained 50 pools that contained clones able to hydrolyze tributyrin, but not tricaprylin or triolein, indicating that the clones produced esterases and not true lipases. The three positive pools that displayed large halos most rapidly were selected for further analysis. The positive clones were isolated from the positive pools and the fosmids were extracted. From the restriction patterns of the fosmids (data not shown), we concluded that the three clones contained different DNA fragments. To evaluate whether the esterase activity was encoded on the fosmid containing the insert, we reintroduced it into *E. coli* strain EPI300™-T1^R with electroporation. The enzymatic activity was confirmed, demonstrating that the esterase activity was encoded by the genomic region carried in the fosmid.

3.2.2 Cellulolytic activity

We identified two pools that could hydrolyze cellulose. Two pools that grew in M9-C minimal medium with Avicel® as the sole carbon source were identified. Both clones were restreaked on M9-C minimal medium with Avicel® and the presence of cellulase-producing clones was confirmed.

3.2.3 Mn oxidation

Interestingly, three pools displayed a brownish-red precipitate when grown in medium used to detect manganese peroxidase (Figures 2a and 2b). However, the expected phenotype of a manganese-peroxidase-producing clone (a sky-blue color when hydrogen peroxide is added) was not observed. The positive clones were then identified in each pool (Figures 2c and 2d) and the corresponding fosmids were purified. The three fosmid preparations displayed similar restriction profiles (data not shown), suggesting that the three clones carried fosmids with similar DNA inserts. Therefore, we selected one of these fosmids for further assay. *Escherichia coli* strain EPI300™-T1^R transformed with this fosmid produced a brownish precipitate in Mn-supplemented medium, confirming that the genetic information contained in the fosmid was responsible for the observed activity.

To determine whether the precipitate was produced in a reaction with the ABTS in the medium, we evaluated the phenotype in media with and without ABTS. The brownish-red precipitate was even present in the absence of ABTS, supporting previous results indicating that the activity was

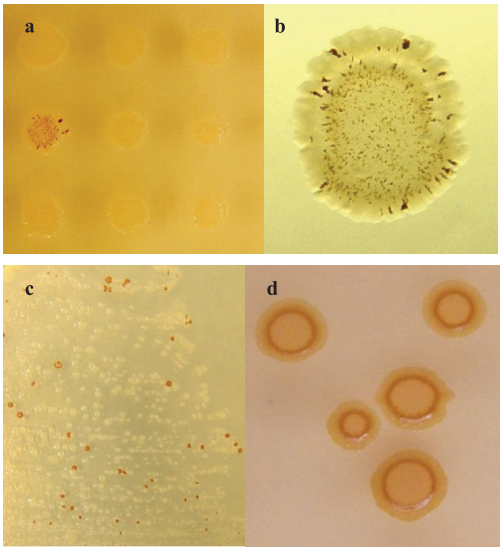

Figure 2 Selection of positive clones with manganese oxidase activity. **a**, Screening the library on different substrates in a 48-pin array suitable for replicate plating. The array shows one pool, among nine, displaying a brownish precipitate. **b**, Magnification of Figure a showing in detail the library pool with the brown precipitate. **c**, Restreaked positive pool in the appropriate screening medium for the isolation of a positive clone. A few brown colonies were apparent among the white colonies. **d**, Isolation of a positive clone in specific medium. All the colonies display the brown precipitate.

not a manganese peroxidase. We evaluated the phenotype in media with and without added manganese and found that the precipitate was only generated in medium supplemented with Mn^{2+} (data not shown). This is the expected phenotype of a manganese-oxidase-producing clone. To determine whether the observed brown color was attributable to manganese oxide, LBB, which specifically reacts with Mn^{3+}–Mn^{7+}, was added on top of the colonies[22-23]. As shown in Figure 3, when LBB solution was added to the colonies displaying the brownish-red precipitate, they turned blue, whereas colonies without the fosmid did not change color. These findings support the notion that this precipitate resulted from a manganese oxidase activity encoded on the fosmid.

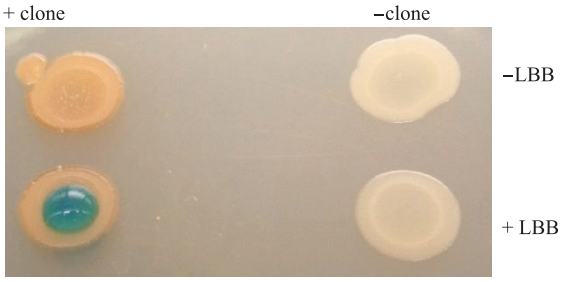

Figure 3 Confirmation of manganese oxidase activity using leucoberbelin blue I reagent. Four bacterial colonies were spotted in a 2 × 2 array (two dots per row, two dots per column) on an LB plate containing 0.5 mM $MnCl_2$. In the left column, clones displaying the brown precipitate phenotype (+ clone). In the right column, clones displaying no such phenotype (– clone). In the upper row, no LBB was added on top of the colonies (–LBB). In the lower row, 5 μL of LBB solution was added on top of the colonies (+LBB). Upper left colony displays the brown precipitate phenotype, whereas the upper left colony is white. Lower left colony (brown) turns LBB blue, indicative of the presence of manganese oxides. In the lower right colony, LBB is colorless, indicating no manganese oxidase activity.

4 Discussion

In this study, we generated a metagenomic library containing about 52 000 clones from an environmental sample collected from a pristine area of the Antarctic Peninsula. Because each clone had an insert estimated to be 35–40 kb, the library contained almost 2 Gb of genetic information. If we assume that the average size of a bacterial genome is 4 Mb and that all the metagenomic DNA was of bacterial origin, the library would contain about 500 bacterial genome equivalents (Table 1). It has been predicted that eukaryotic DNA, although it is present in a very small fraction of the environment (estimated to be less than 0.1% of total cell numbers), represents about 90% of the total environmental DNA because eukaryotic genomes are larger than prokaryotic genomes[24]. We cannot exclude the possibility that eukaryotic DNA was present in the metagenomic library, and further studies are required to evaluate the bacterial diversity present in the sample. However, the large metagenomic library generated was acceptable for a functional study. In fact, we detected 50 lipolytic pools, two pools capable of hydrolyzing CMC, two pools displaying growth on Avicel® as the sole carbon source, and three pools with manganese oxidase activity. Expressed differently, these results show that of the 1 700 pools tested, 2.89% of them displayed lipolytic activity, 0.115% cellulolytic activity, 0.174% manganese oxidase activity, and 0.115% were able grow on Avicel® as the sole carbon source.

When Berlemont et al. functionally screened a BAC metagenomic library generated from Antarctic soil[10], they found that 0.044% of clones (14 of 31 818 clones evaluated) displayed esterase/lipolytic activity and 0.12% (11 of 9 167) displayed cellulase activity. Twice as many lipolytic clones were isolated from our meltwater library than from the previously reported soil library. *A priori*, it is unlikely that similar percentages of metabolic activities would be identified in metagenomic libraries generated from soil and meltwater. However, it is improbable that the meltwater environment requires extensive lipolytic activity for bacterial survival. A plausible explanation of this difference may be that lipase-producing clones were overrepresented in our metagenomic library. An alternative explanation is that the strategy used for library construction in this study favored the isolation of *E. coli* with extracellular esterase activity.

We also report here, for the first time, a manganese-oxidase-producing clone isolated with a functional metagenomic strategy. Although we did not intentionally look for this activity, we characterized these manganese-oxidase-producing clones because there is considerable academic interest in this enzyme. It has been suggested that the Mn oxides present in nature mainly arise from biological Mn^{+2} oxidation and that bacteria play a key role in the biogeochemical cycling of this metal[23]. Mn^{2+}-oxidizers are found in phylogenetically diverse groups and are present in a great variety of environments[25]. Manganese oxides provide bacteria with protection against UV light, ionizing radiation, hydrogen peroxide, metal toxicity, and predation[25], so they probably play an important role in the survival of Antarctic bacteria. Most of the manganese oxidases studied belong to the group of multicopper oxidases[23, 26-27], although some heme peroxidases[28] have also been found. The fosmid was sequenced with the Ion Torrent™ technology and interestingly, no homologues of classical manganese oxidases were found, although an open reading frame similar to that of cytochrome c peroxidase was identified (Ferrés, Iriarte et al., personal communication). Studies to identify the gene responsible for the manganese oxidase activity detected are in progress in our laboratory.

In summary, the functional screening of this Antarctic metagenomic library revealed enzymes with potential biotechnological, environmental, and academic relevance. Because the functional metagenomic approach circumvents the bias inherent in genomic approaches, this library should be a useful tool for the discovery of novel genes and proteins.

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