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ORIGINAL ARTICLE

Using skimmed milk agar to functionally screen a gut metagenomic library for proteases may lead to false positives

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Abstract

Aims: Functional screens using skimmed-milk agar to obtain protease activity is a common approach. The aim of this study was to determine the efficacy of this screen to obtain protease activity from a metagenomic library.

Methods and Results: A distal gut metagenomic library was functionally screened using a skimmed-milk agar. The functional screen provided 231 clones generating the characteristic clear halo indicative of protease production. Clone analysis revealed that they were not protease-positive, but expressed glycosidic hydrolases and produced acid, which was responsible for the clear halos.

Conclusions: The current skimmed-milk agar method to obtain proteases is not sufficiently robust to provide a definitive screen. Other- non-protease activities will also give the same clear halo and these would be interpreted as protease positive clones without further analysis. Hence a more robust buffered medium or a specific protein should be used.

Significance and Impact of the Study: Functional screens are a powerful approach to obtaining enzymes from large metagenomic libraries and proteases are a particularly interesting target. The skimmed-milk agar is not sufficiently robust to ensure that only proteases are isolated and in order to save time and money this study has shown that better designed media can aid in the process.

Introduction

Metagenomics offers researchers a route to search for novel bioactive agents and functions from uncultured microbes. The initial approach described by Rondon *et al.* (2000) outlined various functional screens to obtain metagenomic clones which showed a variety of phenotypes. In the current study we were interested in obtaining protease activity from the gut microbiome and implemented a metagenomic approach using a functional screen based on skimmed milk agar (SMA). We successfully obtained clones that were able to generate the characteristic clear halos indicative of protease production. However, subsequent analysis revealed that this phenotype was not because of proteolytic activity in these clones. We will discuss the results of this screen and the actual identity of the clones obtained.

A fosmid metagenomic library (average insert size 40 kb) was constructed from genomic DNA isolated from bacterial cells obtained after fractionation of a homogenized stool sample. Screening of the library for protease activity was performed on Luria-Bertani (LB) agar supplemented with 1% (w/v) skimmed milk, and chloramphenicol (12·5 μg ml^{-1}) using the QPIX2-XT robot. SMA was prepared using a 10% (w/v) stock solution of commercially available nonfat milk powder and LB agar made separately. LB was autoclaved at 121°C for 15 min, while 10% (w/v) milk powder solution was autoclaved at 115°C for 10 min. Exactly 10% (w/v) milk solution was mixed with LB agar to a final concentration of 1% (w/v) while still hot. Plates were incubated for 3 days at 37°C after which time any clone with a clear halo was picked and stored for subsequent analysis. Transposon mutagenesis of positive clones was performed using the *in vitro*

EZ::*Tn*5 system (Epicentre Biotechnologies, Madison, WI, USA). All sequencing was performed by GATC Biotech (Constance, Germany) using primers specific to the inserted transposon.

The initial screen of the metagenomic library identified 231 positive clones, which all showed the characteristic clear halo on SMA. These clones were further tested on a variety of protein substrates (all obtained from Sigma, Dublin, Ireland): casein (azo-casein, 5 mg ml^{-1}), elastin (elastin congo red, 2·5 mg ml^{-1}), fibrin (fibrin blue, 2 mg ml^{-1}), gelatine (nutrient-grade gelatine, 3% w/v), and collagen (azo-collagen, 1 mg ml^{-1}). Substrates were combined with agar at the concentrations indicated and media sterilized by autoclaving at 115°C for 10 min. After incubation at 37°C for 3 days none of the clones showed any ability to degrade these substrates. A subset of the putative protease clones were mutagenized and clones lacking their original phenotype on SMA were selected. The disrupted loci in five mutants derived from clones prot5, prot47, and prot24, were identified by sequencing from the transposon insert. Analysis of this sequence data using tBLASTX revealed that in the majority of mutant clones the transposon had inactivated genes directly involved in cleaving glycosidic bonds, with homology to β-galactosidases or β-glucosidases. No matches to characterized protease enzymes were observed for any mutant.

In response to these results we tested the ability of all 231 putative protease clones to degrade the chromogenic glycoside substrates X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, 80 μg ml^{-1}), S-Gal (3,4-cyclohexenoesculetin-beta-D-galactopyranoside, 3 mg ml^{-1} plus 5 mg ml^{-1} ferric citrate), and indican (indoxyl-beta-D-glucoside, 25 mg ml^{-1}) in LB agar. All substrates were obtained from Sigma. Indican and S-Gal were added to the media prior to sterilization at 121°C for 15 min. X-Gal was added after media sterilization. All clones were all able to degrade X-Gal and liberate the blue dye, 5-bromo-4-chloro-3-indoxyl. A total of 23 clones produced a positive phenotype on indican agar, suggesting β-glucosidase activity in these clones, while 95 were capable of hydrolysing S-Gal. Therefore, a variety of glycoside hydrolyses with differing substrate ranges were isolated on SMA.

The *Eschrichia coli* EPI300 host strain was unable to hydrolyse X-Gal, S-Gal, or indican. The *E. coli* EPI300 host carrying the pCC1FOS vector alone exhibited only very weak activity on X-Gal, S-Gal and Indican, probably because of leaky repression of the intact *lacZ* gene on the vector. Mutants of prot5 and prot47 disrupted in β-galactosidase genes and unable to generate clear zones on SMA were also unable to hydrolyse X-gal. In addition, an alternate set of 64 clones, derived from a screen of the library for cellulolytic activity, were also unable to degrade X-Gal indicating that cloned DNA inactivates all LacZ activity that may arise from the pCC1FOS vector and thus confirms that the activity was because of the cloned metagenomic DNA.

A subset of clones grown in un-buffered LB skimmed milk broth (SMB) all reduced the pH from approximately 7 to 5·6 or lower. In contrast, the host strain *E. coli* EPI300 and *E. coli* EPI300 carrying the pCC1FOS vector alone elevated the pH of the media (Table 1). Transposon mutants of prot5 and prot47 (prot5-ve1 and prot47-ve1, respectively), with disrupted β-galactosidase genes and a negative phenotype on SMA, elevated the pH of the media to levels comparable with that of the *E. coli* EPI300 host and *E. coli* EPI300 carrying the pCC1FOS vector only (Table 1). Analysis of the supernatants from these cultures by high-performance liquid chromatography (HPLC) showed a correlation between pH reduction of the media and generation of acid metabolites (Table 1). The putative protease clones tested removed lactose from the media and produced acetate, while the host strain and the two mutants tested (prot5-ve1 and prot47-ve1) did not reduce lactose and generated considerably lower levels of acetate. Additional analysis of the metagenomic clones and associated mutants on the media containing the acid pH indicator phenol red [SMA or LB agar containing 1% (w/v) lactose, supplemented with 0·25 mg ml^{-1} of phenol red] verified this acid production after overnight incubation at 37°C. From this observation we were able to mimic the clarification of the SMA media by dropping acid on the skimmed milk agar, which results in a clear patch where the acid was placed.

Attempts were also made to develop alternative media for screening metagenomic libraries for protease-producing clones. The commercially available protein substrate azo-casein was combined with LB agar at a final concentration of 5 mg ml^{-1} and sterilized by autoclaving for 10 min and 115°C. Pure cultures of protease-producing *Pseudomonas aeruginosa* and *Bacillus cereus* were used to validate this media and generated distinct clear zones against the pale orange background of the media after overnight incubation, indicating azo dye release and diffusion as a result of casein degradation. Fosmid clones originally identified as protease-positive on SMA were also tested on azo-casein agar. None of the original 231 clones isolated using SMA produced a positive phenotype on azo-casein agar.

The application of metagenomics provides a viable route to obtain novel whole metabolic pathways, which are functionally active. However, to identify the desired clones within a library robust functional screens are required, otherwise valuable time and resources can be wasted characterizing false-positives. We have embarked

Table 1 Characterization of protease-positive clones derived from metagenomic library screening on 1% w/v skimmed milk powder (SMA) and associated mutants

Strain/clone/media	SMA clearing*	Degradation of X-Gal*	pH of culture media†	HPLC analysis‡	
				Lactose	Acetate
SMB	N/A	N/A	6·95	14·42	1·28
EPI300	–	–	7·82	13·3	8·0
EPI300::pCC1FOS	–	–/+	7·81	12·00	6·05
Prot5	+	+	5·29	4·15	30·9
Prot17	+	+	5·61	BLD	33·12
Prot35	+	+	5·38	BLD	33·9
Prot47	+	+	5·57	1·64	14·77
Prot103	+	+	5·54	BLD	32·62
Prot192	+	+	5·47	BLD	36·17
Prot5-ve1	–	–	7·57	12·79	12·29
Prot47-ve1	–	–	7·84	12·862	6·515

HPLC, high-performance liquid chromatography; X-Gal, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside.

*Strains were grown on Luria-Bertani (LB) agar with 1% (w/v) SMA and supplemented with 80 μg ml^{-1} of X-Gal as appropriate. N/A, not applicable; –, negative phenotype; +, positive phenotype; –/+, weak-positive phenotype.

†All strains were cultured overnight in LB broth plus 1% (w/v) SMA with shaking. After incubation, the cells were removed by centrifugation and the pH of the supernatant was measured.

‡All strains were cultured overnight in LB broth plus 1% (w/v) SMA with shaking. Culture supernatants were obtained by filtration of overnight cultures though 0·02-μm filters (Nalgene) and levels of lactose, lactate and acetate were measured; the values are presented as mmol l^{-1} concentrations and any levels which are below the limits of detection level are shown as BLD.

on a project to identify functions performed by the human gut microbiome and obtain active clones from a large metagenomic library. To date many clones have been isolated which carry out functions of relevance to the gut ecosystem. The ability to degrade proteins was one such function we wished to obtain and we employed the well-established SMA screen to identify clones with this activity. This media has been used previously in metagenomic projects (Lorenz and Eck 2005) and pure culture studies (Pailin *et al.* 2001) as a means to identify proteolytic activity. As we show here this media is not entirely suitable for this purpose, however, it was very effective in obtaining glycoside hydrolases. What we also found striking is the current lack of proteases obtained in metagenomic projects, one has been cited in a recent review (Lorenz and Eck 2005), but the original work remains unpublished. In another study correlating extracellular polysaccharides (EPS) production and protease activity in lactic acid bacteria, the association described may be because of milk sugar utilization and acid metabolite production, rather than EPS-bound proteinases as suggested by the authors (Pailin *et al.* 2001). In conclusion, to search the gut microbiome for novel proteases either a well-buffered milk agar should be used or ideally the pure protein of interest, e.g. casein.

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