RESEARCH PAPER

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Inhibition of the growth of *Bacillus subtilis* DSM10 by a newly discovered antibacterial protein from the soil metagenome

Mark M O'Mahony[1], Ruth Henneberger[2], Joseph Selvin[3], Jonathan Kennedy[1], Fiona Doohan[4], Julian R Marchesi[5,6], and Alan D W Dobson[1,*]

[1]School of Microbiology and Marine Biotechnology Center; Environmental Research Institute; University College Cork; Cork, Ireland; [2]Institute of Biogeochemistry and Pollutant Dynamics; ETH; Zürich, Switzerland; [3]School of Life Sciences; Pondicherry University; Puducherry, India; [4]School of Biology and Environmental Science; University College Dublin; Dublin, Ireland; [5]Cardiff School of Biosciences; Cardiff, UK; [6]Centre for Digestive and Gut Health; Imperial College London; London, UK

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A functional metagenomics based approach exploiting the microbiota of suppressive soils from an organic field site has succeeded in the identification of a clone with the ability to inhibit the growth of *Bacillus subtilis* DSM10. Sequencing of the fosmid identified a putative β-lactamase-like gene *abgT*. Transposon mutagenesis of the *abgT* gene resulted in a loss in ability to inhibit the growth of *B. subtilis* DSM10. Further analysis of the deduced amino acid sequence of AbgT revealed moderate homology to esterases, suggesting that the protein may possess hydrolytic activity. Weak lipolytic activity was detected; however the clone did not appear to produce any β-lactamase activity. Phylogenetic analysis revealed the protein is a member of the family VIII group of lipase/esterases and clusters with a number of proteins of metagenomic origin. The *abgT* gene was sub-cloned into a protein expression vector and when introduced into the *abgT* transposon mutant clones restored the ability of the clones to inhibit the growth of *B. subtilis* DSM10, clearly indicating that the *abgT* gene is involved in the antibacterial activity. While the precise role of this protein has yet to fully elucidated, it may be involved in the generation of free fatty acid with antibacterial properties. Thus functional metagenomic approaches continue to provide a significant resource for the discovery of novel functional proteins and it is clear that hydrolytic enzymes, such as AbgT, may be a potential source for the development of future antimicrobial therapies.

Introduction

In recent years, metagenomic approaches have increasingly been employed to access the uncultured and uncultivable majority of microorganisms present in the environment, which is estimated to be more than 99% of the existing prokaryotes in soil systems.[1] Metagenomic analyses, such as large-scale shotgun sequencing, have to date been applied to various environments and have led to the discovery of novel species, and even the elucidation of novel pathways and community-specific metabolism.[2-7] Apart from sequencing-based approaches, metagenomic libraries can also be screened for the presence and expression of specific functions and activities, also known as gain-of-function, and a range of novel biocatalysts, many with potential industrial applications, as well as small molecules with novel bioactivities, have already been discovered using such functional metagenomics based techniques.[8-16] With its extremely high, but still largely underexplored and unexploited, microbial diversity and abundance, soil is one of the richest potential sources for novel biocatalysts and novel natural products.[17-20] In recent years, functional metagenomic analyses has led to the discovery of a wide range of enzymes and bioactive metabolites, e.g. antimicrobial agents, from the soil microbiota.[21-23] Soils that suppress plant disease, or so called suppressive soils, are known to exert their antagonistic function through their soil microbiota;[24] particularly through the presence of phytopathogenic antagonistic (antibiotic) activity.[25] Indeed it has recently been reported that upon attack by fungal pathogens plants exploit the microbial consortia from soil to protect themselves against infection.[26] Thus suppressive soils which show a natural suppression of growth of certain plant pathogens are likely to be a valuable source for the discovery of novel bioactive compounds.

Metagenomic approaches have also been employed to assess the bacterial natural product biosynthetic diversity of different soil microbial communities.[27] From this work it appears that

*Correspondence to: Alan Dobson; Email: a.dobson@ucc.ie
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prokaryotic natural product biosynthesis biodiversity is potentially much larger than we have previously thought from culture dependent based approaches. Indeed a wide variety of natural products including indigo, indirubin,[28,29] turbomycin A, turbomycin B,[30] violacein,[31] palmitoylputrescine,[32] norcardamine, terragines A-E[33] and tetarimycin A[34] among others, have in the past been isolated from environmentally derived metagenomic libraries. In addition with the advent of improved heterologous host expression systems in *Streptomyces* spp., in different *Proteobacteria* such as *Agrobacterium tumefaciens*, *Escherichia coli*, *Caulobacter vibrioides*, *Pseudomonas putida*, *Burkholderia graminis* and *Ralstonia metallidurans* and in *Saccharomyces cerevisiae*,[18,35-37] an increasing number of bioactive small molecules with potential biopharmaceutical applications are being discovered on an on-going basis. Examples include the pentacyclic polyketide erdacin,[35] the fluostatins[36] the antitumor substance BE-54017,[14] the indolotryptoline antiproliferative agent (borregomycin A) and the dihydroxyindolocarbazole anticancer/antibiotics (borregomycins B-D)[38] together with the antibiotics fasamycins A and B.[39]

Thus given the potential of suppressive soils containing microbiota with antimicrobial and anti-phytopathogenic activities, we employed a functional metagenomics-based approach in an effort to identify novel antibiotic activities from the microbiome of soils from an organic field site. This approach resulted in the cloning of a β-lactamase-like gene, *abgT*, with an ability to inhibit the growth of *B. subtilis* DSM10.

Results

Screening of the soil library

Approximately 14000 clones were screened for antimicrobial activity against *Pseudomonas aeruginosa* PA01 and *Bacillus subtilis* DSM10, and a single clone, TO-T-020-P12, was found to clearly inhibit the growth of *B. subtilis* DSM10 (**Fig. 1**). This clone was subjected to further analysis and was found to consistently produce a zone of inhibition against *B. subtilis* DSM10 in overlay assays. The clone did not, however, appear to have the ability to inhibit *P. aeruginosa* PA01. Furthermore the clone TO-T-020-P12 did not inhibit growth of *B. subtilis* DSM10 in the absence of arabinose, which was responsible for inducing fosmid replication to multiple copy numbers. The TO-T-020-P12 fosmid was isolated and a fresh stock of *E. coli* EPI300™ T1 cells was retransformed with the fosmid and activity was confirmed. *E. coli* EPI300™ T1 clones containing pCC1FOS with the fosmid control DNA supplied with the library production kit were used as a negative control and consistently failed to produce any activity against either *B. subtilis* DSM10 or *P. aeruginosa* PA01.

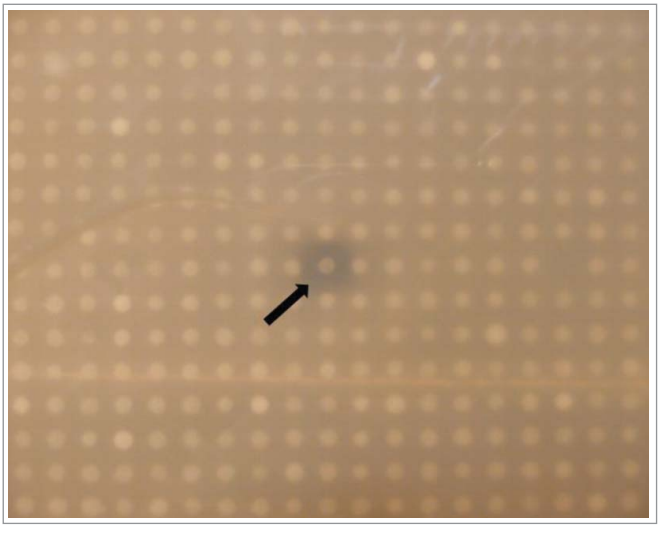

Figure 1. Screening of the soil metagenomic library. Metagenomic clones were overlaid with soft agar containing *B. subtilis* DSM10. Arrow indicates clone TO-T 020 P12 producing a zone of growth inhibition.

Sequencing and annotation of fosmid

The TO-T-020-P12 fosmid was sequenced by Roche 454 pyrosequencing, and assembly of the resulting sequences produced a contig of 42137 bp, with a G-C content of 60.5%. Further examination of the sequence for vector contamination yielded an insert size of 35277 bp (GenBank accession no. JX846920). The insert sequence was analyzed for putative ORFs using both the FGENESB and the MetaGene programmes. While each program predicted a slightly different number of ORFs, in general there was substantial agreement between the main ORFs present (**Table S1**). Each ORF was putatively annotated using blastP searches for homologous proteins and the predicted annotations were further refined by manual examination with the final annotation selected based on evidence that the proteins appeared to be a better fit and more closely-related. A total of 27 ORFs were putatively annotated (**Table 1**, **Fig. 2**). The genes appeared to encode for a range of proteins with potentially diverse functions including proteins involved in hydrolysis, oxidation/reduction, biosynthesis, membrane transport, transcriptional regulation and a number of proteins of as yet unknown function. The putative annotations for each ORF listed in **Table 1** reflect the top matches after blastP searches. The vast majority of the species associated with these proteins appear to be environmental isolates, particularly from soil and aquatic habitats, and include members of the following bacterial phyla; *Proteobacteria*, *Firmicutes*, *Acidobacteria*, *Bacteroidetes* and *Verrucomicrobia*. A number of the putative ORFs were found to be associated with the bacterium Ellin514, with 4 adjacent putative proteins (Genes 9–12) appearing to share homology with proteins from this bacterium. This strain, also known as *Pedosphaera parvula* Ellin514, is an obligate aerobic bacterium, which was isolated from pasture soil in Victoria, Australia, and belongs to the *Verrucomicrobia* phylum whose members are common in terrestrial environments.[40] Attempts were made to further determine the phylogenetic origin of the metagenomic DNA using MEGAN, however the results did not predict any taxonomic origin below the *Proteobacteria* phylum level, and with only 32% of the ORFs assigned to this phylum it is difficult to postulate more precisely the origin of the metagenomic DNA in clone TO-T-020-P12.

Transposon mutagenesis and identification of insertion site

Two mutants, TO-T-020- P12 E2 and TO-T-020-P12 F2, were obtained following screening of transposon mutants which

Table 1. Putative annotation of the open reading frames from fosmid TO-T 020 P12

ORF	Strand	Length (aa)	Putative Proteins	% Coverage / Identity	E value	Accession No.
1*	−	434	hypothetical protein Lcho_1379 [*Leptothrix cholodnii* SP-6]	98/32	1e-34	YP_001790412.1
2	+	413	hypothetical protein Mettu_3900 [*Methylobacter tundripaludum* SV96]	78/41	4e-82	ZP_08782479.1
3	+	296	glucose-1-phosphate thymidylyltransferase [*Leptothrix cholodnii* SP-6]	97/81	9e+172	YP_001789661.1
4	+	291	type 11 methyltransferase [*Candidatus Desulforudis audaxviator* MP104C]	56/33	8e-10	YP_001717291.1
5	+	483	polysaccharide biosynthesis protein [*Beggiatoa* sp PS]	95/32	1e-69	ZP_01999860.1
6	+	234	transferase hexapeptide repeat containing protein [*Pelosinus fermentans* JBW45]	82/43	3e-51	EIW47873.1
7	+	97	hypothetical protein [*Anaeromyxobacter* spFw109–5]	77/40	4e-12	YP_001378438.1
8	+	532	AMP-dependent synthetase/ligase [*Paracoccus denitrificans* PD1222]	97/46	2e-146	YP_915093.1
9	+	308	eight transmembrane protein EpsH [bacterium Ellin514]	88/44	1e-61	ZP_03627452.1
10	+	665	multi-sensor signal transduction histidine kinase [bacterium Ellin514]	99/45	0e+00	ZP_03627457.1
11	+	456	two component, sigma54 specific, transcriptional regulator, Fis family [bacterium Ellin514]	98/68	0e+00	ZP_03627458.1
12	−	210	2-deoxy-D-gluconate-3-dehydrogenase [bacterium Ellin514]	99/29	2e-04	ZP_03627456.1
13	−	755	TPR repeat-containing protein [uncultured bacterium 92]	96/45	0e+00	ADC35860.1
14	−	293	hypothetical protein Cyan7822_3123 [*Cyanothece* spPCC 7822]	82/41	4e-57	YP_003888352.1
15	−	230	HAD family hydrolase [*Geobacter lovleyi* SZ]	93/39	2e-42	YP_001951850.1
16	−	204	hypothetical protein HFX_2454 [*Haloferax mediterranei* ATCC 33500]	85/43	3e-39	YP_006350126.1
17	−	588	acyl-CoA dehydrogenase [*Carboxydothermus hydrogenoformans* Z-2901]	98/49	0e+00	YP_360435.1
18	+	167	DinB family protein [*Solitalea canadensis* DSM 3403]	94/37	9e-25	YP_006254353.1
19	−	390	3-ketoacyl-CoA thiolase [*Candidatus Chloracidobacterium thermophilum* B]	99/69	0e+00	YP_004862742.1
20	−	213	α/β hydrolase [*Coxiella burnetii* RSA 493]	92/41	5e-48	NP_820749.1
21	−	270	α/β hydrolase [*Rubrobacter xylanophilus* DSM 9941]	87/31	1e-13	YP_642926.1
22	−	766	3-hydroxyacyl-CoA dehydrogenase [*Candidatus Chloracidobacterium thermophilum* B]	99/48	0e+00	YP_004862740.1
23	−	267	ABC-2 type transporter [*Chloroflexus aurantiacus* J-10-fl]	99/33	4e-33	YP_001633913.1
24	−	330	ABC transporter-like protein [*Roseiflexus castenholzii* DSM 13941]	91/48	6e-79	YP_001430568.1
25	−	346	hypothetical protein [*Desulfosporosinus meridiei* DSM 13257]	52/31	1e-04	YP_006621226.1
26	−	444	β-lactamase [*Turneriella parva* DSM 21527]	86/55	2e-137	YP_006439821.1
27*	−	234	pyruvate/2-oxoglutarate dehydrogenase [*Candidatus Chloracidobacterium thermophilum B*]	100/64	1e-95	YP_004863213.1

*Partial ORFs.

had lost the ability to inhibit growth of *B. subtilis* DSM10. The transposon insertion sites were determined by bidirectional sequencing and both mutants were found to have insertions at the 3′ end of ORF 26 approximately 55 bp from the stop codon. Putative annotation of the fosmid revealed that ORF 26 showed similarity to a β-lactamase protein (1). This ORF was subsequently designated as gene *abgT* and the results indicated that transcription of this gene had been disrupted and therefore that it was potentially playing a role in the observed antibacterial activity.

Analysis of the *abgT* gene

The *abgT* gene which is 1335 bp yields a predicted protein of 444 amino acids, with a molecular mass of 47.2 kDa. The protein has a theoretical pI of 9.22 and an instability index of 30.65, suggesting that it is a stable protein. The ORF is located toward the 3′ end of the insert sequence and is adjacent to a partial ORF with similarity to a pyruvate/2-oxoglutarate dehydrogenase (**Fig. 2**). The gene appears to have a GTG start codon with an almost perfect Shine-Delgarno consensus sequence located 6 bp upstream. Analysis of the promoter region suggests that

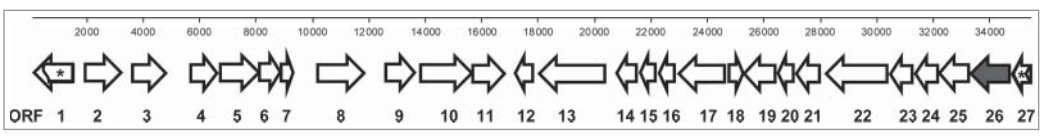

Figure 2. Schematic representation of putative open reading frames. Putative open reading frames were identified in the 35277 bp DNA insert of fosmid clone TO-T-020-P12. The arrows represent the position and direction of transcription of each open reading. The numbers correspond to the ORFs listed in **Table 1**. The *abgT* gene (ORF 26 -**Table 1**) is highlighted in a darker color . Arrows containing * represent partial open reading frames.

this gene may be under the control of its own promoter, with putative -10 (tggcatact) and -35 (gtgtcg) regions beginning 226 bp upstream from the proposed start codon. Further analysis of the protein revealed a potential signal peptide of 25 amino acids (VTAANSLSRILLALALGVTPHVAQA), suggesting that the protein may be secreted. The predicted amino acid sequence of the AbgT protein contains a β-lactamase conserved domain (pfam00144) and has highest identity, of up to 55%, to several proteins classified as putative β-lactamases, with highest identity to a β-lactamase from *Turneriella parva* DSM 21527. Alignments of the AbgT protein sequence with other β-lactamase like proteins, Class C β-lactamases and representative of the different lipase family groups was performed and subsequent phylogenetic analyses revealed that AbgT is a member of the family VIII group of lipases/esterases (**Fig. 3**).[41,42] This group contains the Class C β-lactamases and a number of β-lactamase like proteins, though the exact function of many of these proteins has yet to be biochemically determined.

Lipase and β-lactamase activities

To determine if the protein possessed any potential lipase activity the original clone TO-T- 020-P12 and Tn5 mutant TO-T-020-P12 E2 were incubated on plates containing 1% tributyrin. Clone TO-T-020-P12 produced lipolytic activity (**Fig. 4A**), though the observed activity was quite weak compared to a metagenomic lipase clone which had previously been isolated in our laboratory and determined to have high levels of activity.[13] Transcriptional disruption of the *abgT* gene resulted in a loss of lipolytic activity as the mutant strain TO-T-020-P12 E2 failed to produce any zone of clearing on tributyrin, even after prolonged incubation. As the protein displayed similarity to β-lactamase type proteins the chromogenic nitrocefin assay was used to determine if there was any significant β-lactamase activity present. Both the original clone TO-T-020-P12 and the mutants TO-T-020-P12 E2 and TO-T-020-P12 F2 were tested for activity, using ampicillin resistant cells as a positive control. Cells containing ampicillin resistance genes produced a red color when tested with the nitrocefin as expected. However, the metagenomic clones failed to produce a similar reaction under the conditions tested and therefore it was concluded that AbgT does not possess true β-lactamase activity (**Figure S1**). The overlay assays with *B. subtilis* DSM10 were also performed in conjugation with the nitrocefin assay to confirm that antibacterial activity was observed as expected under the growth conditions used.

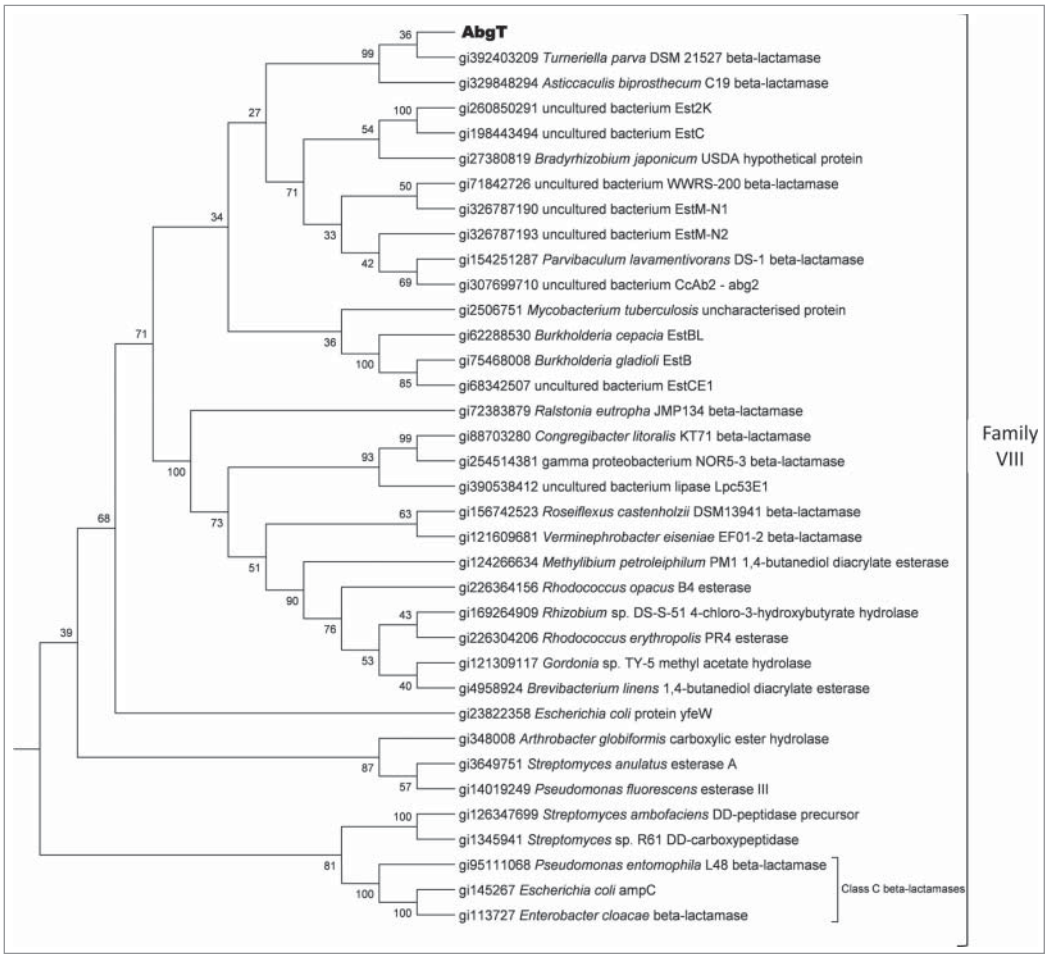

Figure 3. Phylogenetic analysis of the deduced AbgT amino acid sequence. The tree contains proteins closely related to AbgT, putative β-lactamases, Class C β-lactamases and lipase family members. The tree represents the Family VIII lipase cluster and is rooted to the other lipase family groups.

Restoration of antibacterial activity in the *abgT* disrupted mutants

To confirm that the β-lactamase-like gene was indeed involved in the observed antibacterial activity the *abgT* gene was cloned into the expression vector pBadMyc-HisA to create pBadMyc-HisA-AB-RH1. The original clone TO-T 020 P12 and both mutants TO-T-020-P12 E2 and TO-T 020 P12 F2 were transformed with the recombinant vector and the clones were subsequently overlaid with *B. subtilis* DSM10 (**Figure 4B**). The clone TO-T-020-P12 showed clear ability to inhibit the growth of *B. subtilis* DSM10, but as the results indicate when the *abgT* gene was disrupted in the TO-T-020-P12 E2 and TO-T-020-P12 F2 mutants both were unable to inhibit the growth of *B. subtilis* DSM10 strain (**Figure 4B**; 2 and 3). However, complementation of the TO-T-020-P12 E2 and TO-T-020-P12 F2 mutants

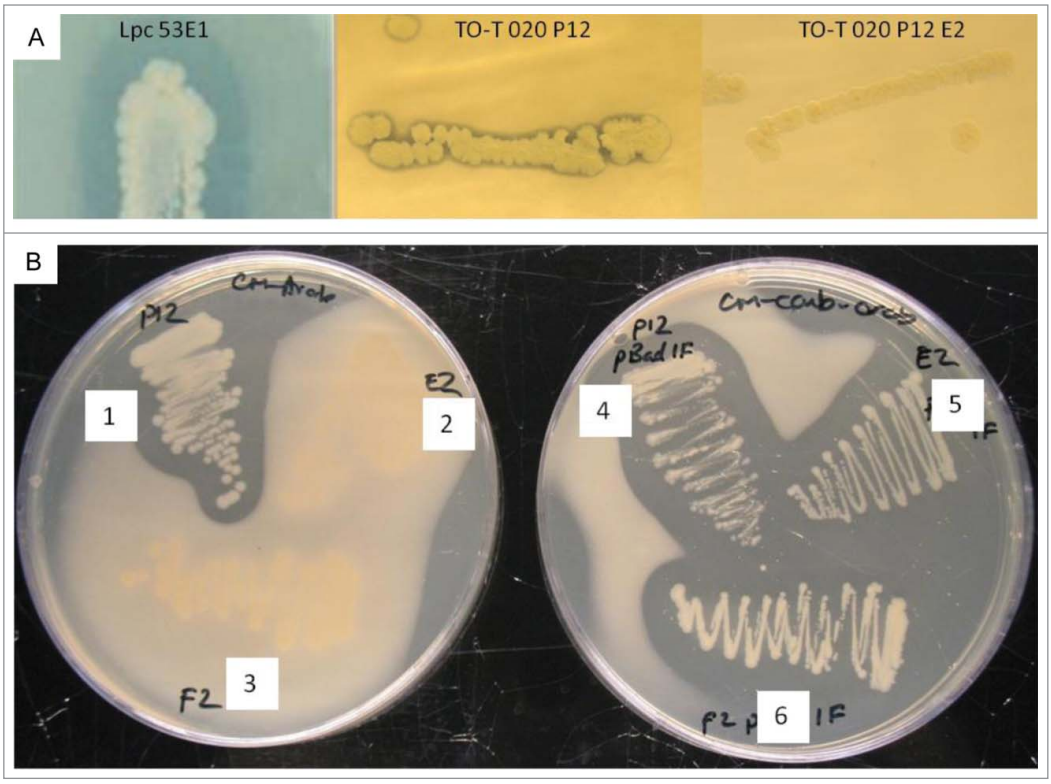

Figure 4. Lipolytic and antibacterial activity of the *abgT* gene. (**A**) Clone TO-T-020-P12 produced weak lipolytic activity on agar plates containing 1% tributyrin. When the *abgT* gene was disrupted by Tn5 insertion in TO-T 020 P12 E2 lipolytic activity was lost. Metagenomic lipase Lpc53E1 was previously characterized in our laboratory and was used for comparison of lipolytic activity. (**B**) Random mutagenesis of fosmid TO-T 020 P12 produced 2 mutants unable to inhibit the growth of *B. subtilis* DSM10. Both mutants regained the ability to inhibit growth after complementation of the disrupted *abgT* gene. (1) TO-T- 020-P12 (2) TO-T-020-P12 E2 (3) TO-T-020-P12 F2 (4) TO-T-020-P12 with pBadMyc-HisA-AB-RH1 (5) TO-T-020-P12 E2 with pBadMyc-HisA-AB-RH1 (6) TO-T-020-P12 F2 with pBadMyc-HisA-AB-RH1.

with the vector borne *abgT* restored the ability of each mutant to inhibit growth (**Figure 4B**; **5** and **6**). As a control, each strain was also transformed with the pBadMyc-HisA vector lacking an insert and the presence of the vector did not affect the inability of the mutants to inhibit *B. subtilis* DSM10 growth. Thus the *abgT* gene product is clearly responsible for the observed anti- *B. subtilis* DSM10 activity. To confirm that a protein of the correct size was being produced from the expression vector the His-tagged AbgT protein was purified from the total soluble protein fraction of cell lysate derived from arabinose induced *E. coli* EPI300^{TM} T1 cells containing pBadMyc-HisA-AB-RH1. Staining of the SDS-PAGE gel revealed a protein with a mass just below 50kDa, which correlates well with the predicted *in silico* molecular mass of AbgT (**Fig. 5**).

Discussion

We report here on the cloning of the *abgT* gene, isolated following the functional screening of a soil metagenomic library; the product of which clearly inhibits the growth of *B. subtilis* DSM10. The AbgT protein while not possessing any β-lactamase activity itself was found to possess a high level of identity with several putative β-lactamases, with highest identity to a β-lactamase from *Turneriella parva* DSM 21527. Strains of *T. parva* have previously been isolated from contaminated culture medium, tap water and from the uterus of a sow.[43] AbgT also displayed similarity to a number of other β-lactamase proteins, including β-lactamases from *Asticcacaulis biprosthecum* C19, *Verrucomicrobiae* bacterium DG1235 and *Muricauda ruestringensis* DSM 13258.

Functional screening of fosmid libraries associated with the marine sponge *Cymbastela concertrica* has identified a novel antibacterial protein, Abg 2, which appears to also have similar characteristics to the AbgT protein.[44] Both showed antibacterial activity against *Bacillus* strains, contain β-lactamase conserved domains and show similarity to the same proteins in the SwissProt database (**Table 2**). Despite these similarities there was only moderate pairwise homology (33%) between these 2 proteins at the amino acid level. In addition the predicted AbgT protein sequence displayed a weak similarity to some lipase/esterase enzymes including a lipase/esterase from *Hydrogenophaga* sp. PBC and a putative esterase from *Methylomicrobium alcaliphilum*. Furthermore analysis of the predicted AbgT protein sequence using the SwissProt database suggested a protein that potentially possessed hydrolytic activity (**Table 2**). The protein showed modest homology to an esterase from *Burkholderia gladioli* and to the YfeW protein from *E. coli* and *Salmonella* species and appears to be similar to type C β-lactamases, which share sequence characteristics with members of the group VIII family of lipases. Recent work in our laboratory has identified a halo-tolerant lipase from the metagenome of a marine sponge which also belongs to this group.[13] Members of this group are known to possess a conserved S-X-X-K amino acid motif in their active site, with a conserved Y amino acid downstream, and analysis of the AbgT protein sequence revealed that it too contains such conserved regions (**Fig. 6**) and shows similarity to EstB from *Burkholderia gladioli*, an esterase which also contains a β-lactamase fold. No significant β-lactamase activity was detected for this protein and yet catalytic activity was shown to be located within the conserved β-lactamase S-X-X-K motif.[45,46]

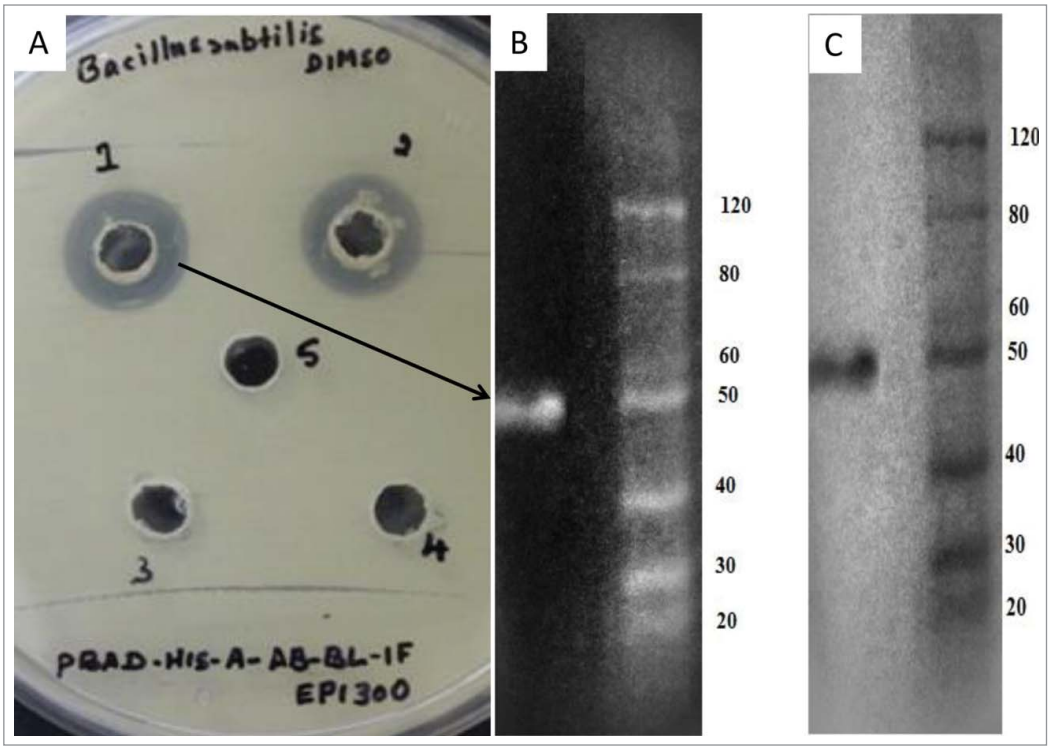

Figure 5. Purification of His_6-AbgT protein. His_6- AbgT was purified from the total soluble protein fraction of cell lysate of *E coli* EPI300™ T1 transformed with the recombinant vector pBadMyc-HisA-AB-RH1. The expressed antibacterial protein was purified from the supernatant by Ni-NTA spin column and resolved by SDS-PAGE on a 10% gel. (**A**) Anti-bacillus activity of the purified Abg protein from cell lysate (1; purified fraction of Ni-NTA column, 2; protein fraction from cell lysate, 3; supernatant fraction, 4; wash from Ni-NTA elution, 5; control with cell free lysate from EP1300 cells). (**B**) Lane 1 stained with Invision His-tag In-gel stain showing the mass of the purified protein at approximately 50 kDa. (**C**) Lane 1 showing the purified protein stained with Coomassie brilliant blue.

We therefore suggest that the AbgT protein may produce hydrolytic activity mediated around the S-X-X-K catalytic site. Furthermore, a protein secreted by the accessory glands of the female sand-fly *Phlebotomus papatasi* has been shown to have both lipase-like activity and antibacterial activity against both Gram-positive and Gram-negative bacteria.[47]

While the exact mechanism by which AbgT produces the observed effects remains to be elucidated it is tempting to speculate that hydrolytic activity may lead to the production of free fatty acids with antibacterial properties. Free fatty acids can be released from lipids by the hydrolytic action of enzymes and have been shown to possess a diverse range of biological activities, including antibacterial properties.[48] Indeed, free fatty acids are thought to play an important role in the innate immune system, defending against potential pathogens particularly in the skin and mucous membranes.[49] The exact mechanism of the antimicrobial action of free fatty acids remains unclear; however, the main target appears to be the cell membrane. There has been a suggestion that free fatty acids may disrupt the electron transport chain or interfere with oxidative phosphorylation, or may lead to increased fluidity of the membrane which can ultimately lead to instability and cell lysis.[48] Moreover in a recent study it has been shown that specific fatty acids can inhibit the growth of *Staphylococcus aureus* by disrupting the cytoplasmic membrane which allows metabolites and low molecular weight proteins to leak from the cell.[50] Thus the AbgT protein may act by releasing fatty acids that lead to disruption of the cell membrane in *B. subtilis* DSM10, resulting in cell death. Another alternative is that the AbgT protein may function in a similar manner to a putative lipase which the Brady group have recently isolated following the functional screening of a *Ralstonia metallidurans* hosted soil metagenomic library.[37] They propose that the anti *B. subtilis* activity displayed by this putative lipase may result from its ability to cleave ester bonds present in the bacterial cell wall.

While the generation of antibacterial free fatty acids is a plausible explanation for the inhibition of the growth of *B. subtilis* DSM10 it should also be noted that a recent study discovered 2 novel esterases from a soil metagenome that were capable of reactivating the antibiotic activity of chloramphenicol from its acetylated derivatives formed by the action of chloramphenicol acetyl tranferases.[51] As chloramphenicol was present in the media and the *B. subtilis* DSM10 strain was sensitive to this antibiotic, the AbgT protein may produce a similar effect. However there was no sequence similarity between AbgT and the 2 esterases identified in the above study and in addition, these esterases were members of the group IV family of lipases rather than the VIII family.

In summary, the data suggests that the AbgT protein is involved in growth inhibition of *B. subtilis* DSM10 and it seems likely that the effects may be mediated by hydrolytic activity. These results suggest not only that functional metagenomic based approaches can provide a significant resource for the discovery of novel functional proteins but that hydrolytic enzymes involved in the release of free fatty acids may be a target for the development of new biomedical therapies.

Materials and Methods

Sampling

Suppressive soil samples were collected from an organic field trial site at the Teagasc Oak Park research facility (Co. Carlow,

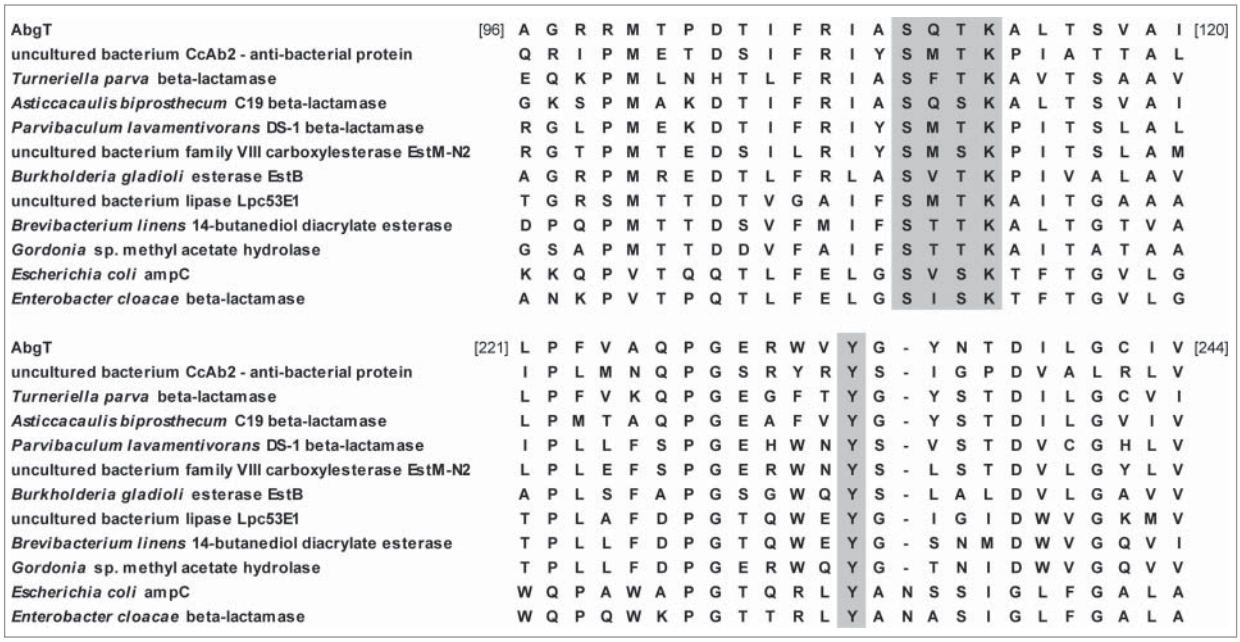

Figure 6. Partial alignment of AbgT with related members of the family VIII group of lipases. The alignment includes; closely related putative β-lactamase proteins from *Turneriella parva, Asticcacaulis biprosthecum* and *Parvibaculum lavamentivorans*; antibacterial protein from CcAb2; esterase and hydrolase enzymes from uncultured bacteria (EstM-N2 and Lpc53E1), *Burkholderia gladioli, Brevibacteroum linens* and *Gordonia* sp; class C β-lactamases from *Escherichia coli* and *Enterobacter cloacae*. Conserved sites S-X-X-K and Y are shaded. The numbers represent amino acids of the AbgT protein.

Ireland; http://www.agresearch.teagasc.ie/oakpark/) that had been in yearly crop rotation for 7 y and had only been fertilized with organic farmyard manure and compost. Samples were taken from a triticale field shortly after harvest. Bulk samples were taken from the surface to a depth of approx. Ten cm using sterile, DNA-free tools (treated with 5% sodium hypochlorite for 30 min prior to washing and autoclaving) and stored in sterile, DNA-free plastic containers. Samples were transported to the laboratory on ice, aseptically fractionated and stored at 4°C until further processing.

Extraction and purification of High Molecular Weight DNA

Metagenomic DNA was extracted from the soil as previously described[52] with the following modifications. Soil was sieved through one stainless steel mesh (mesh size 4 mm). Fifteen grams of freshly sieved soil was mixed with 20 ml preheated (70°C) lysis buffer in 50 ml tubes and incubated at 70°C for 2 hours with thorough mixing by hand every 30 min. After cooling for 30 min at room temperature, the tubes were centrifuged at 4000 × g for 20 min at 4°C and the supernatant was transferred to a fresh tube. The soil pellet was re-suspended in fresh preheated lysis buffer (20 ml) and the extraction procedure was repeated. Supernatants from both extractions were pooled and DNA was precipitated with 0.7 vol isopropanol at −20°C overnight. Precipitated DNA was pelleted by centrifugation at 5000 × g for 60 min at 4°C and the pellet was washed once with 10 ml of 70% ethanol and air-dried. One ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8) was added to the pellet and the high-molecular weight (HMW) DNA was dissolved slowly by incubation at 4°C for several hours with gently regular agitation by hand. The crude HMW-DNA was purified and size-separated by pulsed-field gel electrophoresis (PFGE; at 6 V / cm, 1–25 switch time, 120° angle, 17 hrs, 14°C) in 0.5x TBE (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, pH 8.3) on a 1% PFGE grade agarose gel (Bio-Rad Laboratories). For size comparison, each gel contained a PFGE DNA Molecular marker (MidRange II PFG Marker, New England Biolabs). The marker lane and approx. 0.5 cm of the sample DNA lane were cut off and stained for 30 min in 1x SYBR Safe dye (Invitrogen) for visualization. Two gel-slices (approx. One cm) containing unstained DNA of about 30–50 kb and 50–70 kb were cut out and the DNA was electro-

Table 2. Protein similarity searches for AbgT amino acid sequence

ORF	% Identity	Protein	Organism	E Value	Swiss-Prot Accession No.
26 (abgT)	37	Uncharacterized protein Rv1367c/MT1414	Mycobacterium tuberculosis	6e-54	P9WLZ2 /P9WLZ3
	34	Esterase EstB	Burkholderia gladioli	2e-42	Q9KX40
	30	UPF0214 protein YfeW	Escherichia coli O157:H7	7e-31	Q8XBJ0
	30	UPF0214 protein YfeW	Escherichia coli strain K12	2e-30	P77619
	29	UPF0214 protein YfeW	Salmonella enteritidis PT4	2e-28	B5R4I5

eluted out of the gel and concentrated as described by Brady[52] with the following modifications: Electro-elution was performed for 3 h with replacement of running buffer after 90 min; DNA was concentrated to a final volume of approx. 100 μl with Viva-Spin 6 (MWCO 50000) concentrators (Sartorius). Washed and concentrated DNA was dislodged from the membranes by placing the filter upside-down in a clean 50 ml tube and centrifuging for 5 min at 5000 × g. Size and purity was checked by PFGE with the conditions stated above. DNA concentration was determined using the NanoDrop ND-1000 spectrophotometer.

Construction of metagenomic fosmid library

HMW DNA was end-repaired and metagenomic libraries were constructed using the CopyControl™ Fosmid Library Production Kit pCC1FOS (Epicentre Biotechnologies), according to the manufacturer's instructions. Titers were calculated and approximately 4000–5000 *E. coli* EPI300™ T1 fosmid clones were plated onto 20 × 20 cm Luria-Bertani (LB) agar supplemented with 12.5 μg/ml chloramphenicol. The clones were picked with the Genetix Qpix2 XT robotic system (Molecular Devices) into 384-well plates containing LB media with the following supplements: 6.3 g/l K_2HPO_4, 1.8g/l KH_2PO_4, 0.5 g/l sodium citrate dihydrate, 0.09 g/l $MgSO_4.7H_2O$, 6% glycerol. After incubation at 37°C for 18 - 20 hrs, the libraries were stored at -80°C. Twelve clones from the HMW DNA library were randomly picked for determination of average insert size. Isolated fosmids were digested with *Not*I for 3–4 hr and analyzed by Pulsed Field Gel Electrophoresis with the following conditions: 1% agarose in 0.5% TBE, 6 V/cm, 1–25 switch time, 120° angle, 11.5 hrs, 14°C. Induction of the fosmid to multiple copies was performed using 10% arabinose (filter sterilized). Fosmid DNA was extracted using the GeneJET™ Plasmid Miniprep kit (Fermentas) and DNA was eluted in 30 μl of water. To ensure efficient library production the fosmid control DNA supplied with the kit was also subjected to the same reaction conditions as the isolated HMW DNA. *E. coli* EPI300™ T1 clones containing pCC1FOS with cloned fosmid control DNA were subsequently used as a control when screening the soil metagenomic libraries for antimicrobial activities.

Screening of fosmid libraries for antimicrobial activities

Fosmid clones were replicated into fresh 384-well plates containing LB medium, supplemented with 12.5 μg/ml chloramphenicol, and incubated at 37°C overnight. The clones were arrayed on 20 × 20 cm agar plates containing LB medium, 12.5 μg/ml chloramphenicol and 0.01% arabinose using the QPix2 XT robotic system. Plates were incubated at 37°C overnight, followed by further incubation at 25°C for 3–5 d after which the clones were exposed to UV light for 1 min and then overlaid with soft (0.5%) agar containing the following test strains: *Pseudomonas aeruginosa* PA01 and *Bacillus subtilis* DSM10. *P. aeruginosa* PA01 and *B. subtilis* DSM10 were grown to OD_{600} of approx. One.0 and 1.5 respectively and diluted 1:50 for *B. subtilis* DSM10 and 1:100 for *P. aeruginosa* PA01 in soft LB agar before being carefully poured over the metagenomic clones. The plates were incubated overnight and examined for zones of growth inhibition.

Sequencing and annotation of fosmid

Fosmids were sequenced by Roche 454 pyrosequencing. Sequencing and assembly were carried out by the University of Liverpool, Center for Genomic Research. Contigs from the assembly were analyzed for putative open reading frames (ORFs) using the FGENESB-Bacterial Operon and Gene Prediction Program (www.softberry.com) and the MetaGene program.[53] The Basic Local Alignment Search Tool (BLAST) at NCBI (http://www.ncbi.nlm.nih.gov/BLAST) was used to search DNA and protein databases for homologous DNA and protein sequences.[54] Potential ORFs were analyzed using blastP searches against the non-redundant protein sequences database and the SwissProt database. Conserved functional or structural protein domains were identified using the Conserved Domain Database at the NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).55 Sequences upstream of predicted start codons were examined for the presence of bacterial promoters using the BPROM-Prediction of Bacterial Promoters Program (www.softberry.com). The theoretical parameters of putative proteins were calculated using the ExPASy ProtParam tool.[56] Protein sequences were also examined for signal peptides using the SignalP 4.0 server.[57] Multiple sequence alignments and phylogenetic analyses were conducted using MEGA version 4.1 software.[58] Evolutionary trees were constructed using the Neighbor-Joining method with a bootstrap test of 1000 replicates. Attempts were made to predict the potential phylogenetic origin of the fosmid DNA using MEGAN.[59]

Transposon mutagenesis of fosmids

Fosmids were subjected to mutagenesis using the EZ-Tn5™<TET-1> Insertion kit (Epicentre Biotechnologies), according to the manufacturer's instructions. The mutant mini-library was plated on LB medium containing 12.5 μg/ml chloramphenicol and 10 μg/ml tetracycline. The clones were hand-picked into liquid media in 96-well plates, incubated overnight and subsequently replicated onto solid LB medium containing 12.5 μg/ml chloramphenicol and 0.01% arabinose to induce the fosmids to multiple copies. The plates were then incubated overnight at 37°C, followed by 2 further overnight incubations at 25°C and overlaid with the *B. subtilis* DSM10 as described above. Mutants that appeared to have lost the ability to inhibit growth were selected for further analysis. The insertion site of the EZ-Tn5 transposon was mapped using bidirectional sequencing with primers TET-1 FP-1 (5′ GGG TGC GCA TGA TCC TCT AGA GT 3′) and TET-1 RP-1 (5′ TAA ATT GCA CTG AAA TCT AGA AAT A 3′).

Detection of lipolytic and β-lactamase activities

Lipolytic activity was detected by plating the clones on LB medium containing 1% tributyrin (Sigma-Aldrich). The plates were incubated at 37°C overnight, followed by further incubation at 25°C for up to 7 d during which the plates were examined regularly for zones of clearing. The chromogenic β-lactamase

substrate nitrocefin (Calbiochem) was used to detect β-lactamase activity. Briefly, a 1 mM working solution of nitrocefin was prepared according to the manufacturer's instructions. One drop of the nitrocefin solution was placed directly on the surface of a colony and the development of a red color was used as an indication of a positive result. In addition the colonies were also tested for activity by emulsifying the colony into one drop of the nitrocefin solution on the surface of a glass slide.

Sub-cloning of the *abgT* gene and complementation of the transposon mutant

The *abgT* gene was amplified from the fosmid TO-T 020 P12 using the primers AB-BL F Pag (5′ TAT TAC TCA TGA CCG CTG CGA ACT CAC TG 3′) and AB-BL R IF Hind (5′ ATC ATT AAG CTT TCT TCC GTG GCG GAC 3′). Restriction sites were mis-primed (underlined above) into the 5′ end of the primers to allow insertion of the gene into the expression vector pBadMyc-HisA (Invitrogen). The gene was inserted under the control of the arabinose inducible P_{BAD} promoter and the stop codon of the open reading frame was removed to allow a C-terminal fusion with the poly-histidine region of the vector. PCR was performed using *Pfu* DNA polymerase and the PCR product (~1.3 kb) was purified from a 1% agarose gel and subsequently digested using the restriction enzymes *Pag*I and *Hind*III at 37°C for 90 min. The pBadMyc-HisA vector was digested using *Nco*I and *Hind*III using similar conditions. The digested products were purified and ligated overnight at 16°C with T4 DNA ligase (Fermentas). *E. coli* EPI300™ T1 cells were transformed with the ligation mixture and plated on LB medium containing 100 μg/ml carbenicillin. The presence of the insert was confirmed by colony PCR and restriction digestion. Transposon mutant clones were subsequently transformed with the purified recombinant vector.

Purification of His-tagged AbgT protein

E. coli EPI300™ T1 cells containing the recombinant pBadMyc-HisA vector were grown in LB medium containing 100 μg/ml carbenicillin until mid-log phase and then induced with 0.01% arabinose for 4 h. The cells were harvested by centrifugation, re-suspended in lysis buffer (50 mM of NaH_2PO_4, 300 mM of NaCl, 10 mM imidazole, pH 8.0) and incubated on ice for 20 minutes. Cells were then disrupted by sonication in ice with 3 5 second pulses at high intensity and the supernatant was collected after centrifugation. The His-tagged protein was purified by Ni-NTA spin column with varying concentration of imidazole (20- 250 mM) used in the elution buffer to maximise the amount of purified product in a single elute. The purified protein was dialyzed and lyophilized before being resolved by SDS–PAGE.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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