Universidade do Minho
Escola de Engenharia

Semana da Escola de Engenharia
October 24 - 27, 2011

IDENTIFICATION OF AN ANTIMICROBIAL GENE CLUSTER IN *Serratia* Sp.

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INTRODUCTION

Antibiotic resistance is a growing concern in clinical settings. As more pathogens acquire resistance to previously effective antibiotics, we face serious challenges controlling detrimental bacterial proliferation. The quest for novel and effective antibiotics has led scientists to re-examine bacteria as a source of these compounds. In this work, we isolated a strain of *Serratia* that produces an extremely potent antimicrobial agent, effective against a broad range of pathogens. Using random transposon mutagenesis, we obtained mutants defective in antibiotic production. Several transposon insertions occurred in a large gene cluster predicted to encode enzymes involved in the synthesis of what appears to be a novel hybrid polyketide-non-ribosomal peptide (PK-NRP). We are currently completing the sequence analysis of the gene cluster and determining the structure of the compound.

METHODS, RESULTS & DISCUSSION

Isolates obtained from biofilms of a dairy processing plant were screened for production of antimicrobial. For that, the cell-free supernatant of stationary-phase cultures was tested on several Gram positive and negative pathogens. One of the many positive hits, a *Serratia sp.*, was selected for more in-depth studies, due to the potency of the secreted compounds, that displayed antimicrobial activity against *Helicobacter pylori*, *Campylobacter coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Enterobacter sakazakii*, *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, among others.

To identify genes involved in the biosynthesis of the antimicrobial, transposon mutagenesis was carried out. Mutants were grown on minimal medium, over a layer of sterile membrane that allowed the diffusion of compounds while acting as a barrier to cells. After removal of the membrane, the cell-free, but exudate-rich, agar plates were overlaid with a lawn of *Escherichia coli* K12, to test for loss of halo (Figure 1).

Several random transposon mutants, which no longer displayed antimicrobial activity, were isolated.

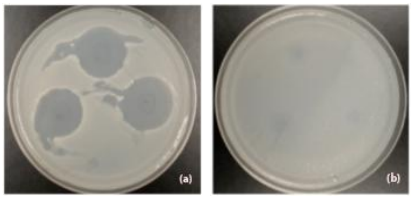

Figure 1: Screen for loss of antimicrobial production. Wild type (a) and transposon mutant (b) exudate, overlaid with *E. coli* K12. Three replicates were grown per plate for the assay.

The genes that were interrupted by transposons were mapped by arbitrary PCR and the sequences obtained were used in a BLAST search against the non-redundant protein database. The initial sequences appeared to be encoding genes homologous to polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS).

PKS/NRPS gene clusters tend to be quite large, spanning tens of thousands of base pairs. To facilitate mapping of the entire biosynthetic region, we fully sequenced the genome of the *Serratia* sp. isolate. The regions upstream and downstream of the genes with transposon insertions were screened for open reading frames (ORF) using bioinformatic tools (DNASTAR Lasergene 8). The edges of the biosynthetic gene cluster were thus determined, based on predicted function (Figure 2). Genes encoding proteins involved in the compound translocation are transcribed from both strands. However, the enzymes and other proteins predicted to be involved in the assembly of this molecule are transcribed from the plus strand.

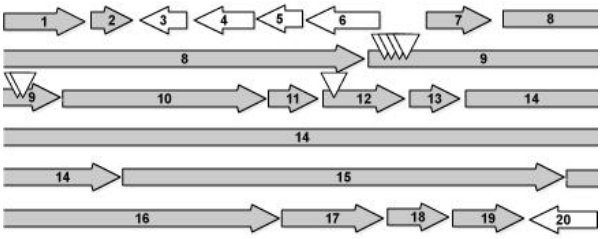

Figure 2: Map of the antimicrobial's biosynthetic gene cluster, of 52.174 bp. The open reading frames and corresponding transcription direction are represented by arrows; the triangles represent locations of the transposon insertions that abolished antimicrobial production.

From the genome analysis it was possible to confirm that the antimicrobial compound being made is a PK-NRP hybrid. PKS and NRPS genes are organized in clusters and result from the coordination and multistep activity of the enzymes arranged in these assembly lines. The core components include betaketoacyl synthases, which are responsible for polymerizing fatty acids - acyl-CoA thioesters - into polyketides and amino acid adenylation domains are responsible for amino acid processing for incorporation in the compound (NRPS), as aminoacyl AMP anhydrides.

Simplifying, betaketoacyl synthases are responsible for polymerizing fatty acids - acyl-CoA thioesters - into polyketides. Amino acid adenylation domains are responsible for amino acid processing for incorporation in the compound (NRPS), as aminoacyl AMP anhydrides.

The type and order of the domains - the catalytic units of these enzymes - are directly responsible for when and which monomer is activated and added to the molecule, which transformation (post-translation modification) it will undergo, and the type and length of product that will eventually be made. Nevertheless, in the case of hybrid PK-NRP compounds as this, a full prediction of the outcome is particularly tricky, as it includes two distinct types of molecules.

The proteins predicted to be transcribed from the 52.714 base pairs (bp) were characterized for their domains (Table 1), using several databases available online: ClusScan, Web-CD Search Tool, SMART, NRPSpredictor, NRPSpredictor2, PKS/NRPS Analysis Web-site and SBSPKS. The structure of this antimicrobial is currently being solved by Nuclear Magnetic Resonance, in collaboration with the Clardy Lab at Harvard Medical School (US).

Table 1: Proteins predicted to be encoded by the biosynthetic gene cluster, and respective domains for post-translation modification.

ORF	Predicted Protein	Domain
1	Pyridoxal-dependent decarboxylase	Pyridoxal decarboxylase
2	Hypothetical Protein	No domains
3	Phosphopantetheinyl Transferase	ACPS
4	HlyD	RND_mfp C
5	ABC Transporter	FtsE
6	Membrane Spanning Subunit	FtsX
7	Hypthetical Protein	No domains
8	Betaketoacyl Synthase	KS; KS_C; AT; PP binding (PCP); KR; DH
9	Betaketoacyl Synthase	KS-KS_C; KS-KS_C; TE
10	PfaD	NPD_FabD; AAT_1
11	3-Oxoacyl-Reductase	ACP-Reductase
12	Hypothetical Protein	SDR_e1
13	NAD(P)binding Protein	Nitrilase
14	aa Adenylation Domain	A (?); PCP; C; A (Phe); PCP/T; C; A (Asn); PCP; C/E: C/Cy; A (Asn); PCP/T
15	aa Adenylation Domain	C/E; A (Thr/dht); PCP; C; A (Val); PCP
16	6-Deoxyerythronolide-B-Synthase	KS-KS_C; AT; KR/E; ACP
17	Condensation-Domain-Containing Protein	C; E/Cy
18	Polyamine-Transporting ATPase	P-Loop NTPase; Methyltransferase
19	ABC-2-type Transporter	ABC-type multidrug transport system, permease component
20	Hydrolase family protein	Esterase

A(aa) =Adenylation (amino acid activated); ACPS=holo-[acyl-carrier-protein] synthase; RND_mfp C=Resistance-Nodulation-Cell Division_Membrane Fusion Protein; FtsE=Cell Division ATP-binding protein; FtsX=protein insertion permease; KS=ketosynthase; KS_C=C-terminus; AT=aminotransferase; PP_binding= Phosphopantetheine attachment site; KR=ketoreductase; DH=dehydrogenase; TE=thioesterase; ACP=Acyl Carrier Protein; NPD=Nitropropane dioxygenase; AAT_1=Aspartate Aminotransferase; SDR_e1=Short-chain dehydrogenases/reductases; PCP=Peptidyl Carrier Protein; C=Condensation; Cy=Cyclization; E=Epimerization; T=Thiolation.

AUTHOR BIOGRAPHY

Sara Cleto was born in Vila Nova de Gaia, Portugal. She carried out her undergraduate studies at University of Porto and did internships in The Netherlands and Italy (1999-2004). After graduating, Sara also worked as a medical writer and scientific translator/editor. In University of Minho and under the supervision of Professor Maria João Vieira, she researched on biofilm formation and eradication. Currently she is finishing her Ph.D. under the supervision of Professor Roberto Kolter (Harvard Medical School, USA) and Professor Maria João Vieira (University of Minho, Portugal). In Boston (USA), Sara has also volunteered for several science outreach programs and actions bridging scientists, biotech companies and universities from Europe and the US.

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