Poster No. C-1424

Transposon Directed Insertion-Site Sequencing (TraDIS) to Elucidate the Mode of Action of the Antimicrobial Arenicin-3 (Arn-3)

Amy K. Cain[1], Christine J. Boinett[1], Lars. Barquist[2], Søren Neve[3], Johnny X. Huang[4], Alysha G. Elliott[4], Mark S. Butler[4], Mark A. Blaskcovich[4], Julian. Parkhill[1], Matthew A. Cooper[4]

1. Wellcome Trust Sanger Institute, London, United Kingdom, 2. Institute for Molecular Infection Biology, University of Würzburg, Würzburg, Germany, 3. Adenium Biotech, Copenhagen, Denmark, 4. Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia

m.cooper@uq.edu.au
Phone: +61-7-334-62044

Background

Arenicin-3 (Arn-3) is an antimicrobial peptide (GFCWYVCYRNGVRVCYRRCN) from the lugworm *Arenicola marina*. It exhibits potent and rapid *in vitro* and *in vivo* bactericidal activity against a range of multiple resistant pathogenic Gram-negative bacteria. However, the mode of action (MoA) of Arn-3 remains elusive. We used Transposon Directed Insertion Sequencing (TraDIS; Langridge et al., 2009), to assay all non-essential genes simultaneously for involvement in Arn-3 resistance and sensitivity in two G -ve species to identify the MoA.

TraDIS Methodology

Dense Tn*5* libraries consisting of 220,000 and 600,000 unique mutants were constructed in *Klebsiella pneumoniae* strain Ecl8 and *Enterobacter cloacae* strain NCTC9394. Triplicate pools were grown in ¼ MIC Arn-3 and in LB, then sequenced on an Illumina HiSeq2500 platform. Reads were mapped onto a reference using SMALT and insertion depth per gene calculated. Genes likely to contribute to increased Arn^R or Arn^S had significantly less or more recovered Tn*5* insertions after selection than the LB control (Figure 1).

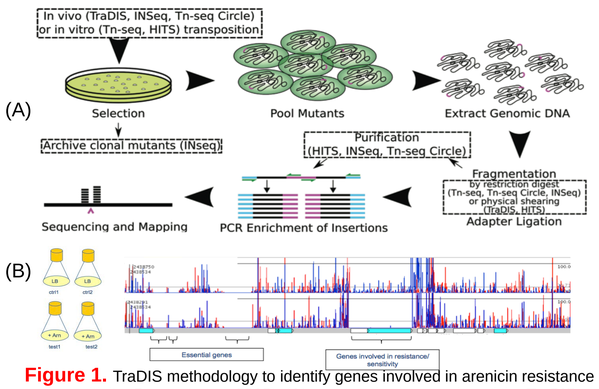

Figure 1. TraDIS methodology to identify genes involved in arenicin resistance and sensitivity (Barquist et al, 2013). (A) construction/processing of the TraDIS libraries (B) Interpretation of TraDIS data via insertion site

Results summary

Genes with different numbers of insertions (changing mutant populations) across *K. pneumoniae* and *E. cloacae* grown +/- Arn-3 are presented in Table 1.

- 3 genes (*waaE/G/Q*) were identified as involved in Arn-3 sensitivity (blue in Table 1), indicating that Arn-3 targets specific steps in LPS synthesis (both core and lipid A), consistent with predicted MoA from previous phenotypic studies (Poster No. F-256).
- 8 genes contributed to resistance (pink in Table 1), including a 2-component regulator important in resistance to peptide antibiotics a multidrug efflux exporter and further OM lipoprotein.

Table 1. Summary of genes involved in arenicin resistance and sensitivity

Gene	logFC[1]	Function
Genes with decreased insertions (Arn-3 resistance)		
macB	-4.94	ABC-type antimicrobial peptide transport system, ATPase component
phoP	-4.78	Transcriptional regulatory protein PhoP
macA	-4.6	ABC-type antimicrobial peptide transport system, efflux transporter
tolC	-4.39	Type I secretion outer membrane protein, TolC
phoQ	-4.35	Sensor protein PhoQ; Signal transduction histidine kinase
yfgL	-3.11	Outer membrane assembly lipoprotein YfgL
slyB	-2.94	Outer membrane lipoprotein SlyB; Outer membrane lipoprotein
rfaH	-1.98	Transcriptional activator RfaH
Genes with increased insertions (Arn-3 sensitivity)		
waaG	2.01	Putative lipopolysaccharide core biosynthesis protein RfaG
waaQ	2.04	Lipopolysaccharide core biosynthesis glycosyltransferase RfaQ
waaE	3.35	Glycosyl transferase, group 2 family protein

logFC: Log fold changes
1: P-value < 0.0001

Arn-3 sensitivity genes

All genes detected are part of the *waa* (or *rfa*) cluster which mainly encodes proteins for synthesis of the core LPS, specifically glycosyltransferases (Fig 2)

WaaQ is the transferase for HepIII (Regue et al., 2001)

WaaG is the transferase for Glcl

WaaE is the transferase for HepI (Izquierdo et al., 2002)

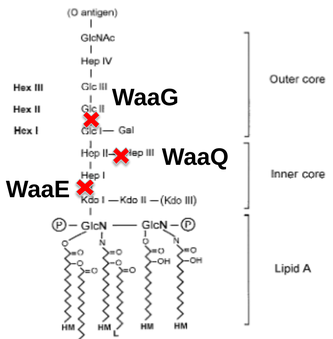

Figure 2. Positions of effected genes in pathways of UDP-GlcNAc (Liu and Reeves, 1994)

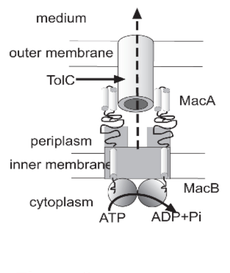

Figure 3. MacAB/TolC efflux resistance mechanism (Lu and Zgurskaya, 2013)

Arn-3 resistance genes

MacAB/TolC The drug transporter consists of the periplasmic membrane-fusion protein MacA, the inner membrane transporter MacB and the outer-membrane channel TolC (Figure 3). All 3 components are required for macrolide resistance. Expression of the *macA/B* operon is repressed by PhoP/Q

PhoPQ is a regulatory system that controls over 40 genes, and this regulon varies greatly between species. SNPs in *phoQ* leads to polymyxin resistance

SlyB is a major OM lipoprotein of the outer membrane and contributes to the integrity of the cell envelope. *slyB* mutatations show increased sensitivity to EDTA and SDS and increased membrane permeability It is regulated by phoPQ, but also negatively regulates PhoP

YfgL is an outer-membrane lipoprotein that is part of a complex that promotes folding and assembly into the outer membrane (exact function not yet fully known)

RfaH regulates ~10 *waa* (aka *rfa*) genes for LPS synthesis and regulation occurs via antitermination of mRNA transcription at specific rho-independent terminators.

Conclusion

Using TraDIS, a clear and specific MoA for Arn-3 was suggested for two pathogenic G -ve species (Figure 4). Arn-3 resistance occurs via non-specific mechanisms where the outer membrane proteins YfgL and SlyB, controlled by the PhoPQ regulator, provide resistance to arenicin by changing the permeability/integrity of the membrane and via the active efflux system of macAB/TolC, also regulated by PhoPQ. Sensitivity was narrowed down to glycoslytranferases that add sugars to the LPS core, indicating a potential target for Arn-3.

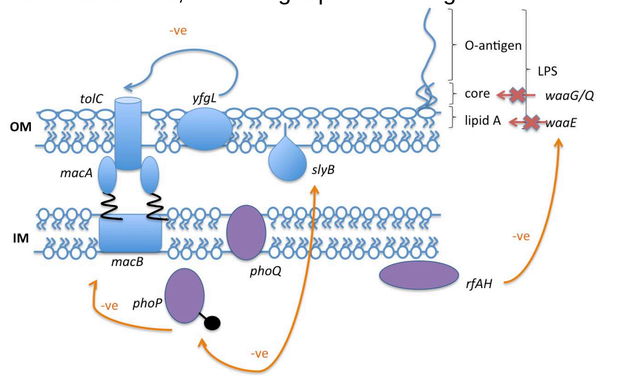

Figure 4. Predicted mechanisms of arenicin action and resistance. Blue proteins are the OM proteins involved in resistance. Purple are regulators involved in resistance. Red are possible arenicin targets.

Acknowledgements

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