Prevalence of Fst-like toxin–antitoxin systems

Toxin–antitoxin (TA) systems are widespread in Gram-negative bacteria, and all systems identified to date encode a toxic protein and an unstable antitoxin, which may be in the form of either an antisense RNA (type I) or a second protein (type II). The enterococcal plasmid pAD1-encoded TA system (*par*), encoding the Fst toxin, was the first type I TA system identified in Gram-positive bacteria (Weaver *et al.*, 1996). In a recent issue of *Microbiology*, Weaver *et al.* (2009) identified an additional eight pAD1-like TA systems. Individual *fst*-like genes were identified on the chromosomes of *Enterococcus faecalis*, *Lactobacillus casei* and *Staphylococcus saprophyticus* strains, plasmids from *E. faecalis*, *Lactobacillus curvatus* and *Staphylococcus aureus*, and a phage from *Lactobacillus gasseri*. It was also hypothesized that the small size of Fst-like toxins may cause the failure of other members of the family to be recognized and annotated. We have now addressed this issue through iterative TBLASTN searching of the translated NCBI nucleotide sequence database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Fst-like toxins were found to be prevalent in a diversity of Gram-positive bacteria. More than 200 additional toxins were detected in the database, and sequence similarity between previously identified Fst peptides and new family members in pairwise alignments was shown to be statistically significant using PRSS ($E < 0.02$; Pearson, 1996). All are 27 to 35 residues in length and are predicted to contain a hydrophobic transmembrane domain. New Fst family members were found to be encoded chromosomally by *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Lactobacillus helveticus*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus brevis*, *Streptococcus pneumoniae*, *Streptococcus equi*, *Streptococcus suis*, *Streptococcus thermophilus*, *Streptococcus mutans* and

Listeria monocytogenes. Additional Fst-like toxins were also found on plasmids from *Enterococcus faecalis*, *Enterococcus faecium*, *Staph. aureus*, *Staph. epidermidis*, *Staphylococcus warneri*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *Lactococcus lactis*, *Macroccoccus caseolyticus* and *Carnobacterium divergens*.

Analysis of the DNA sequence encompassing representatives of the newly identified Fst coding sequences from the various species revealed the presence of characteristic features shown to be important for function of the prototype pAD1 TA system (Greenfield & Weaver, 2000; Greenfield *et al.*, 2000). Specifically, in addition to expression sequences for the toxin, they each possess sequences necessary for expression of an antisense RNA antitoxin, including a potential promoter closely resembling the σ^{70} consensus and a bidirectional terminator, converging towards the end of the toxin gene (Fig. 1). Thus, the resulting toxin mRNA and antitoxin possess a complementary thermodynamically stable stem–loop structure derived from the terminator; the loop residues have been shown to be important for interactions between the transcripts that result in the inhibition of pAD1 Fst toxin expression (Greenfield *et al.*, 2001). Likewise, the directly repeated sequences DRa and DRb are readily identifiable. These tandemly arranged repeats overlap the toxin start codons, and are repeated downstream of the bidirectional terminators within each antitoxin gene, so as to provide a region of complementarity between the single-stranded 5'-tail of the antitoxin and the translation initiation region of Fst mRNA.

Other intramolecular helices in the Fst mRNA have recently been shown to be important in the prototype pAD1 system: a 5' stem–loop structure that sequesters the Fst ribosome-binding site (comprising RBS and anti-RBS sequences), causing translational inhibition (Shokeen *et al.*, 2008); and an upstream helix formed between complementary sequences at each end of the transcript (5' UH and 3' UH) that contributes to the stability of the toxin mRNA *in vivo* (Shokeen *et al.*, 2009). The presence of sequences corresponding to all of these features in association with the newly identified toxin-coding sequences (Fig. 1) suggests a conserved mechanism of RNA–RNA interaction and control of toxin expression. It is therefore likely that most, if not all, of the Fst-like peptides detected are encoded by functional TA systems. Although the features above are well conserved, the nucleotide sequences of individual systems are quite divergent, even between multiple systems co-existing in the same strain, suggesting that cognate TA components might exhibit specificity.

Fst-like TA systems are particularly prevalent in *Staphylococcus* species, although this may partly reflect the abundance of sequence information available for this genus. All 15 available completely sequenced *Staph. aureus* chromosomes contain at least two distinct systems that are conserved between strains, with some possessing more; MRSA252 has four systems but two are carried by an integrated plasmid. *Staph. epidermidis* RP62A contains five systems in its chromosome, several of which may have resulted from plasmid integration, whereas *Staph. epidermidis* ATCC 12228 was found

Microbiology Comment provides a forum for discussion of scientific issues arising directly from papers published in the journal. The authors of papers under discussion will be offered an opportunity to respond.

Guidelines on how to submit a *Microbiology Comment* article can be found in the Instructions for Authors at <http://mic.sgmjournals.org>

It should be noted that the Editors of *Microbiology* do not necessarily agree with the views expressed in *Microbiology Comment*.

Charles J. Dorman, Editor-in-Chief

Fig. 1. Nucleotide sequence alignment of Fst-like TA systems from different bacterial genera. Efae, *Enterococcus faecalis* V583 (GenBank Accession: AE016830); Cdiv, *Carnobacterium divergens* plasmid pCD3.4 (DQ087597); Mcas, *Macroccoccus caseolyticus* plasmid pMCCL2 (AP009486); Spne, *Streptococcus pneumoniae* TIGR4 (NC_003028); Llac, *Lactococcus lactis* plasmid pSK11L (DQ149244); Saur, *Staphylococcus aureus* MRSA252 (NC_002952); Lbre, *Lactobacillus brevis* ATCC367 (CP000416); Lmon, *Listeria monocytogenes* HCC23 (CP001175); c, chromosome-encoded; p, plasmid-encoded. Conserved features predicted include the toxin-coding regions (grey shading), ribosome-binding sites (RBS, bold with black shading), promoters for the toxin and antitoxin genes (black shading), bidirectional terminators (bold with stem sequences underlined), 5' sequences complementary to the RBS (anti-RBS sequences boxed) that form the SL translational inhibitor structure, and the 5' and 3' UH sequences (bold) that form the nuclease-protective upstream helix (UH) structure. Direct repeats DRa and DRb (arrows), required for antitoxin-mediated repression of toxin translation, are shown with lower-case letters indicating residues of the toxin mRNA and antitoxin RNA that can hybridize using standard RNA-RNA pairing rules.

to carry three chromosomal systems and two plasmid-encoded systems. Fst-like TA systems are commonplace but not ubiquitous on theta-replicating staphylococcal multiresistance and conjugative plasmids, with several, such as VRSAp and pN315, containing two; none were detected on small rolling-circle plasmids. Additionally, the staphylococcal pathogenicity island SaPIbov2, which is involved in biofilm formation (Ubeda

et al., 2003), was found to carry a TA system. Likewise, at least one *fst*-like toxin gene was found in each of the available *E. faecalis* chromosome sequences and on most enterococcal plasmids. New Fst-like systems were also detected in some of the available *E. faecium*, *Enterococcus casseliflavus* and *Enterococcus gallinarum* genome sequences, but these are all unclosed and our analyses suggest that the contigs might be plasmid derived;

however, the possibility that they represent chromosomally integrated plasmids cannot be excluded.

Weaver *et al.* (2009) noted an association between the previously identified chromosomally encoded Fst-like systems and genes involved in carbon metabolism. The newly identified Fst-like TA systems were commonly associated with recognizable mobile genetic elements, or remnants thereof, even when located

chromosomally. However, one of the conserved systems on the *Staph. aureus* chromosome is located between genes that encode a putative ABC transporter and glycerate kinase, whereas the system in *L. monocytogenes* is downstream of a gene encoding a glycosyl hydrolase. The common carriage of Fst-like TA systems on resistance plasmids in particular is potentially significant in terms of the maintenance of antimicrobial resistance in important Gram-positive pathogens.

ACKNOWLEDGEMENTS

Research in our laboratory is supported by grants from the National Health and Medical Research Council of Australia.

Stephen M. Kwong, Slade O. Jensen and Neville Firth

School of Biological Sciences, The University of Sydney, NSW 2006, Australia

Correspondence: Neville Firth
(neville.firth@sydney.edu.au)

Greenfield, T. J. & Weaver, K. E. (2000). Antisense RNA regulation of the pAD1 *par* post-segregational killing system requires interaction at the 5' and 3' ends of the RNAs. *Mol Microbiol* 37, 661–670.

Greenfield, T. J., Ehli, E., Kirshenmann, T., Franch, T., Gerdes, K. & Weaver, K. E. (2000). The antisense RNA of the *par* locus of pAD1 regulates the expression of a 33-amino-acid toxic peptide by an unusual mechanism. *Mol Microbiol* 37, 652–660.

Greenfield, T. J., Franch, T., Gerdes, K. & Weaver, K. E. (2001). Antisense RNA regulation of the *par* post-segregational killing system: structural analysis and mechanism of binding of the antisense RNA, RNAI and its target, RNAII. *Mol Microbiol* 42, 527–537.

Pearson, W. R. (1996). Effective protein sequence comparison. *Methods Enzymol* 266, 227–258.

Shokeen, S., Patel, S., Greenfield, T. J., Brinkman, C. & Weaver, K. E. (2008). Translational regulation by an intramolecular stem-loop is required for intermolecular RNA

regulation of the *par* addiction module. *J Bacteriol* 190, 6076–6083.

Shokeen, S., Greenfield, T. J., Ehli, E. A., Rasmussen, J., Perrault, B. E. & Weaver, K. E. (2009). An intramolecular upstream helix ensures the stability of a toxin-encoding RNA in *Enterococcus faecalis*. *J Bacteriol* 191, 1528–1536.

Ubeda, C., Tormo, M. A., Cucarella, C., Trottonda, P., Foster, T. J., Lasa, I. & Penadés, J. R. (2003). Sip, an integrase protein with excision, circularization and integration activities, defines a new family of mobile *Staphylococcus aureus* pathogenicity islands. *Mol Microbiol* 49, 193–210.

Weaver, K. E., Jensen, K. D., Colwell, A. & Sriram, S. I. (1996). Functional analysis of the *Enterococcus faecalis* plasmid pAD1-encoded stability determinant *par*. *Mol Microbiol* 20, 53–63.

Weaver, K. E., Reddy, S. G., Brinkman, C. L., Patel, S., Bayles, K. W. & Endres, J. L. (2009). Identification and characterization of a family of toxin–antitoxin systems related to the *Enterococcus faecalis* plasmid pAD1 *par* addiction module. *Microbiology* 155, 2930–2940.

DOI 10.1099/mic.0.038323-0

Prevalence of Fst-like toxin–antitoxin systems – author's response

The work of Kwong, Jensen and Firth (above) dramatically expands the pAD1 *par*-like family of RNA-regulated toxin–antitoxin (TA) systems described in our recent study (Weaver *et al.*, 2009). While the primary sequences of these systems are highly diverse, important interacting and regulatory structures are clearly conserved. These results would appear to contradict an earlier report indicating that, while several proteic TA systems are widespread among enterococci, *par*-like sequences are rare (Moritz & Hergenrother, 2007). However, given the sequence diversity of the *par*-like systems identified, it is not surprising that the PCR-based screening approach used by Moritz & Hergenrother (2007) failed to amplify any of the *par*-like loci. This information should be considered in future surveys designed to

determine the distribution of other type I TA systems.

Our work and that described above should also be viewed in the larger context of RNA-regulated, probably membrane-localized, small peptide toxins, which were originally reviewed by Fozo *et al.* (2008). While the number of Gram-positive representatives in their sample was limited, it is now clear that such peptides are widespread in Gram-positive bacteria and are likely to play roles at least as important as their counterparts in Gram-negative bacteria. Finally, while most of the *par*-like loci appear to be associated with mobile elements, the association of some of the chromosomally located loci with genes involved in basic carbon metabolism is intriguing. Since the function of many chromosomally encoded TA systems has been elusive and controversial, it would be worth determining if regulation of these elements is integrated with basic cellular metabolism.

Keith E. Weaver

Division of Basic Biomedical Sciences, Sanford School of Medicine, University of South Dakota, Vermillion, SD 57049, USA

Correspondence: Keith E. Weaver
(kweaver@usd.edu)

Fozo, E. M., Hemm, M. R. & Storz, G. (2008). Small toxic proteins and the antisense RNAs that repress them. *Microbiol Mol Biol Rev* 72, 579–589.

Moritz, E. M. & Hergenrother, P. J. (2007). Toxin–antitoxin systems are ubiquitous and plasmid-encoded in vancomycin-resistant enterococci. *Proc Natl Acad Sci U S A* 104, 311–316.

Weaver, K. E., Reddy, S. G., Brinkman, C. L., Patel, S., Bayles, K. W. & Endres, J. L. (2009). Identification and characterization of a family of toxin–antitoxin systems related to the *Enterococcus faecalis* plasmid pAD1 *par* addiction module. *Microbiology* 155, 2930–2940.

DOI 10.1099/mic.0.039198-0