

## Brief report

# The use of functional genomics for the identification of a gene cluster encoding for the biosynthesis of an antifungal tambjamine in the marine bacterium *Pseudoalteromonas tunicata*

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### Summary

**A large insert library was created in *Escherichia coli* from the DNA of the surface-associated marine bacterium *Pseudoalteromonas tunicata*. Screening of the library for antifungal activity resulted in the detection and identification of a large gene cluster encoding for the biosynthesis of an antifungal tambjamine. A biosynthetic pathway has been proposed based on analysis and annotation of the gene cluster.**

### Introduction

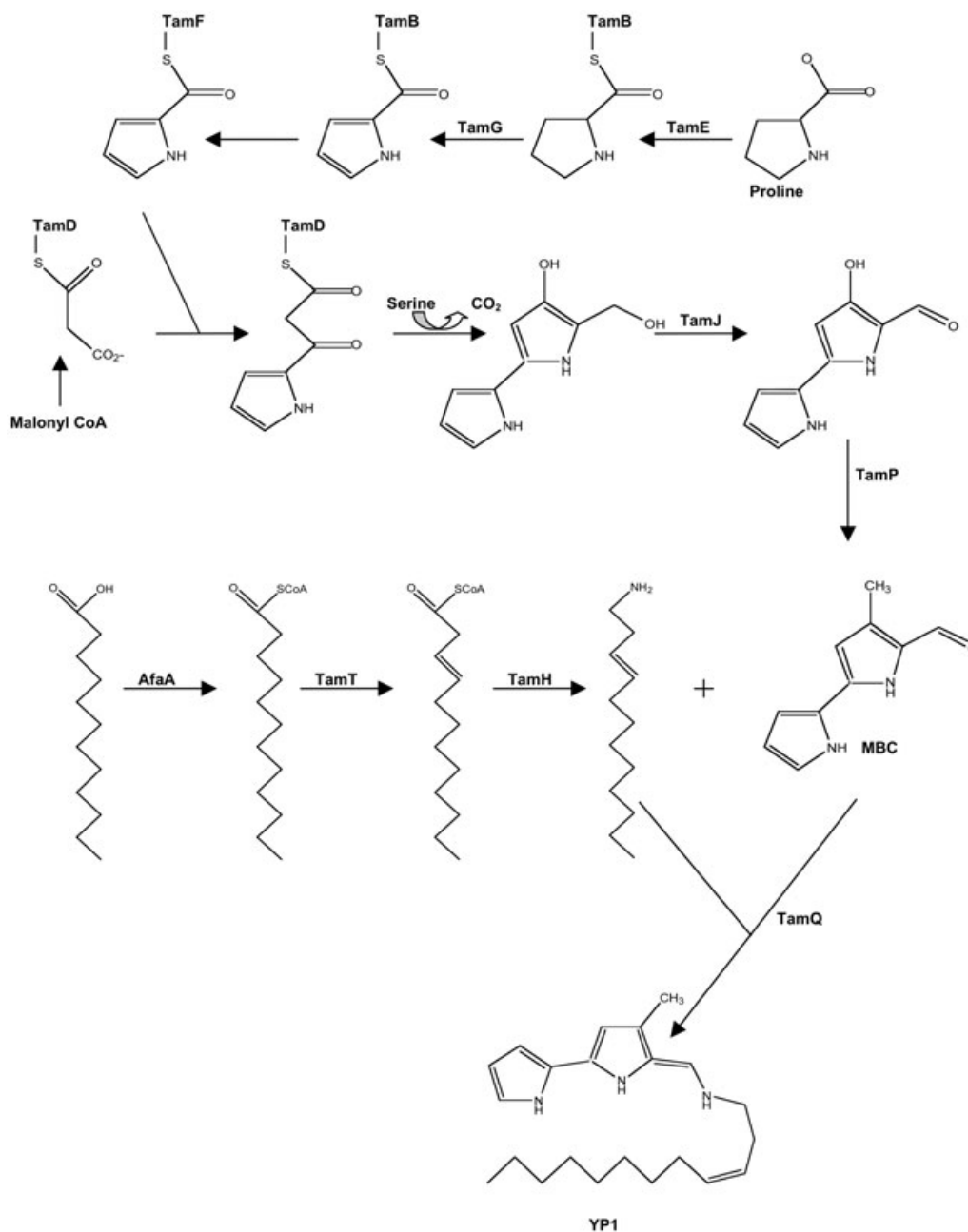
Tambjamins are bioactive compounds from the 4-methoxy-pyrrollic class (Melvin *et al.*, 2001) and have been isolated from several marine invertebrates including nudibranchs (Carté and Faulkner, 1983; Paul *et al.*, 1990), ascidians (Lindquist and Fenical, 1991) and bryozoans (Blackman and Li, 1994). They were found to have characteristics including ichthyodeterrent (Lindquist and Fenical, 1991) and antibacterial (Tanigaki *et al.*, 2002), and these activities have been hypothesized to act as a chemical defence mechanism against natural predators (Carté and Faulkner, 1983; 1986; Paul *et al.*, 1990). While tambjamins and other bioactive compounds clearly play a role in the ecology of the higher organisms from which they are isolated, there has been speculation as to the origin of these secondary metabolites, with increasing

evidence suggesting that many may be synthesized by microbial symbionts (Davidson *et al.*, 2001; Piel *et al.*, 2004; König *et al.*, 2006).

*Pseudoalteromonas tunicata* is a marine bacterium that has been isolated from several locations, including the surface of an adult tunicate *Ciona intestinalis* in Sweden (Holmström *et al.*, 1998) and the surface of the common green alga *Ulva australis* off the east coast of Sydney, Australia (Egan *et al.*, 2000). *Pseudoalteromonas tunicata* produces a range of bioactive compounds with directed activity against algal spores (Egan *et al.*, 2001), marine invertebrate larvae (Holmström *et al.*, 1992), protozoa (Matz *et al.*, 2004), bacteria (James *et al.*, 1996) and fungi (Egan *et al.*, 2002). Antifungal activity has been directly correlated to the production of a yellow pigment (Egan *et al.*, 2002) recently identified as a tambjamine (Franks *et al.*, 2005). Previous studies have shown that production of the active tambjamine, designated YP1 (see Fig. 1), gives *P. tunicata* a competitive advantage in biofilms with marine fungi, both in glass flow cells and on the surface of *U. australis* (Franks *et al.*, 2006). This suggests an ecological role for YP1 production and is consistent with previous hypotheses that *P. tunicata* may aid in the prevention of biofouling on the surface of *U. australis* (Holmström and Kjelleberg, 1999).

There is only one other report of tambjamine production in bacteria, that being from the terrestrial *Streptomyces* strain BE18591 (Kojiri *et al.*, 1993). *Pseudoalteromonas tunicata* is the first known example of a marine bacterium producing a compound from the tambjamine class, which is of particular significance as tambjamins have almost exclusively been isolated from marine sources. In this study we describe the detection and identification of the gene cluster through the use of functional genomics (i.e. the screening of the entire *P. tunicata* genome for antifungal activity in a recombinant *Escherichia coli* background) and postulate a biosynthetic pathway for production of the tambjamine YP1. To our knowledge this is the first description of biosynthetic genes for the tambjamine compound class.

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**Fig. 1.** Proposed biosynthetic pathway of the tambjamine YP1. Proline, malonyl Co-A and serine are incorporated in the synthesis of MBC as described by Williamson and colleagues (2005); TamS (not pictured here) carries out the phosphopantetheinylation of the acyl-carrier protein domains of TamB and TamD. AfaA activates dodecenoic acid, TamT introduces the double bond between the  $\beta$  and  $\gamma$  carbons and TamH transfers an amino group from an amino acid. The resulting dodec-3-en-1-amine is condensed with MBC by TamQ to form YP1.

## Results and discussion

A large insert library was constructed in *E. coli* EPI300 using a fosmid CopyControl™ vector (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. A total of 884 clones (average insert size 35 kb) were screened ( $n = 3$ ) for antifungal activity against *Candida albicans* by means of a soft agar overlay as

described in the study by Lawton and colleagues (1976). Antifungal activity was detected in three independent yellow pigmented clones under plasmid high copy number.

In order to identify the pigment produced by the yellow *E. coli* clones, cells were extracted with chloroform : iso-propanol (80:20, v/v). The extract was dried down and re-suspended in dichloromethane before being analysed

**Table 1.** Open reading frames present on the genome region common to all three yellow *E. coli* clones and the predicted function of the encoded proteins.

ORFs present in fosmid insert	Predicted protein function	Pig and Red proteins with sequence similarity	Transposon mutant pigment/activity
ORF1	Hypothetical protein		+/+
ORF2	Putative phosphopantetheinyl transferase		No mutant obtained
TamA	AMP binding protein		-/-
TamB	Peptidyl carrier protein	RedO, PigG (M)	-/-
TamC	Putative oxidase	RedG	-/-
TamD	HBM synthetase (seryl transferase)	RedN, PigH (M)	-/-
TamE	L-prolyl-AMP ligase	RedM, PigI (M)	-/-
TamF	Pyrrolyl- $\beta$ -ketoacyl ACP synthase	RedX, PigJ (M)	-/-
TamG	L-prolyl-PCP dehydrogenase	RedW, PigA (M)	-/-
TamH	Putative class III aminotransferase	PigE	-/-
TamJ	HBC dehydrogenase	RedV, PigM (M)	-/-
TamK	Hypothetical protein		-/-
TamL	Putative permease		-/-
TamM	Putative permease		-/-
TamN	Putative ABC transporter, ATP binding protein		-/-
TamO	Hypothetical protein		-/-
TamP	HBC O-methyl transferase	RedI, PigF (M)	-/-
TamQ	Terminal condensing enzyme	RedH, PigC	-/-
TamR	Hypothetical protein	RedY, PigK	No mutant obtained
TamS	Phosphopantetheinyl transferase	RedU, PigL (M)	-/-
TamT	Putative dehydrogenase		-/-

Proteins with sequence similarity to those from the Red and Pig clusters are indicated, with Red and Pig proteins involved in MBC biosynthesis marked by an (M). Open reading frames for which transposon mutants were detected and the presence (+) or absence (-) of yellow pigmentation and antifungal activity are also indicated.

by electrospray mass spectroscopy (UNSW Biomolecular Mass Spectrometry Facility). The extract showed a distinct, molecular mass of 356 Da, which is consistent with the mass of YP1 produced by *P. tunicata* (Franks *et al.*, 2005). The molecular mass of 356 Da was not detected in an *E. coli* EPI300 clone that does not produce the yellow pigment (data not shown).

Fosmids from active, yellow clones were sequenced from both ends using EpiFos forward and reverse primers (Epicentre, Madison, WI, USA). Sequencing reactions were analysed at the Automated Sequencing Facility (University of New South Wales). Sequencing reads were aligned to the *P. tunicata* draft genome (GenBank Accession: NZ\_AAOH000000000), and all three fosmid clones were found to contain a common genomic region of approximately 25 kb. This region contains 21 open reading frames (ORFs) of which 19 form a distinct, unidirectional gene cluster with gaps of over 1000 bp on either side. Upstream of the cluster a consensus region for a bacterial promoter was identified using the Softberry BPROM program (<http://www.softberry.com>). These 19 ORFs have been designated the Tam cluster. The ORFs were subjected to extensive annotation using BLAST searches against the NCBI protein database (Altschul *et al.*, 1990), the Manatee annotation platform (The Institute of Genomic Research) and BAYSys (Van Domselaar *et al.*, 2005). The annotation of each ORF is displayed in Table 1.

To further support the boundaries of the Tam cluster, *in vitro* transposon mutagenesis was carried out on one yellow fosmid clone using the EZ-Tn5™ insertion kit according to the manufacturer's instructions (Epicentre, Madison, WI, USA). Yellow and non-pigmented transposon mutants were tested for activity against *C. albicans* as described earlier. Disrupted genes were identified by sequencing from the transposon insert using Tet-1 forward and reverse primers (Epicentre, Madison, WI, USA), and mutants were identified in 18 of the 21 ORFs present. All mutants obtained that were disrupted in ORFs from the predicted Tam cluster lost both pigmentation and antifungal activity while those mutants obtained in genes outside the cluster remained both yellow and active (see Table 1), further supporting the assumption that all 19 Tam ORFs form a distinct transcriptional unit.

Based on the evidence given below we propose a pathway for YP1 tambjamine biosynthesis as displayed in Fig. 1. Of the 19 proteins encoded in the Tam cluster, 12 were found to have high sequence similarity to the Red proteins responsible for undecylprodigiosin synthesis in *Streptomyces coelicolor* A3(2) (Cerdeño *et al.*, 2001) and the Pig proteins involved in prodigiosin biosynthesis in *Serratia* sp. (Williamson *et al.*, 2005) (see Table 1). The three pyrrole rings of prodiginines are structurally related to tambjamins, which are composed of two pyrrole rings with an enamine moiety in place of the third ring. Biosynthetic pathways for the production of prodigiosin and

undecylprodigiosin have previously been proposed based on gene knockout mutants, analysis of accumulating intermediates, genetic complementation and cross-feeding studies (Cerdeño *et al.*, 2001; Williamson *et al.*, 2005). A common feature of both pathways is the formation of 4-methoxy-2,2'-bipyrrrole-5-carbaldehyde (MBC) from proline, serine and malonyl CoA (see Williamson *et al.*, 2005). The condensation of MBC to either 2-methyl-3-n-amylopyrrole (MAP) or 2-undecylpyrrole forms prodigiosin or undecylprodigiosin respectively. Proteins were identified from the Tam cluster with high sequence similarity to all nine proteins assigned a function in MBC synthesis in both the Red and Pig pathways (TamB, D, E, F, G, J, P and S, see Table 1). This provides strong evidence that MBC synthesis also occurs in *P. tunicata* as part of tambjamine biosynthesis.

Previous studies have demonstrated that a disruption of the protein designated AfaA in *P. tunicata* resulted in the loss of antifungal activity (Egan *et al.*, 2000; Franks *et al.*, 2006); however, the precise role this protein plays in the biosynthesis of the tambjamine compound remains unknown. Interestingly *afaA* is not present in the Tam cluster described here. AfaA is predicted to be an acyl-CoA synthetase with high sequence identity to the acyl-CoA synthetase FadD from *E. coli*. It has previously been shown that AfaA and FadD are functionally interchangeable (A. Franks, unpubl. data), which would indicate that the function of AfaA can be replaced by FadD activity during recombinant tambjamine production in *E. coli*. Given its similarity to FadD, AfaA is presumed to be responsible for the activation of long-chain fatty acids, while TamT, a predicted dehydrogenase, may be responsible for the introduction of a double bond into a fatty acyl side chain. Conserved domains for predicted reductase and aminotransferase activity in TamH (data not shown) suggest that this protein may carry out the reduction of the CoA-ester (see Fig. 1) to an aldehyde intermediate, followed by a subsequent transamination to form an amine. The condensation of the resulting dodec-3-en-1-amine and MBC by TamQ, which is homologous to the terminal condensing enzymes from the proposed Pig and Red pathways (Williamson *et al.*, 2005) (see Table 1), would then result in the tambjamine structure of YP1 (see Fig. 1).

Of the remaining proteins in the Tam cluster, TamI, TamM and TamN are predicted to constitute an ABC transporter (two permeases and an ATP binding protein, see Table 1). Phylogenetic analysis of the ATP binding component did not reveal any close clustering with known importers or exporters of the ABC transporter family (data not shown). The presence of a signal peptide sequence in TamK and its proximity to the ABC transporter genes (data not shown) could indicate that this protein acts as the periplasmic substrate binding protein, which would speak towards an import rather than export function for the Tam-

associated ABC transporter. TamC is similar to RedG, which is thought to form cyclic derivatives of undecylprodigiosin via oxidative cyclization (Cerdeño *et al.*, 2001). Given that a cyclic derivative of YP1 is also produced by *P. tunicata* (A. Franks, unpubl. data) it is possible that TamC catalyses this step.

Three proteins are encoded on the Tam cluster for which no biochemical role has yet been assigned. Sequence analysis of TamA shows that it has an adenosine monophosphate binding domain and a phosphopantetheine binding site, while TamO shows no similarity to any characterized proteins or domains. Finally, TamR shows similarity to the uncharacterized RedY and PigK proteins; the presence of these homologues in all three clusters suggests that they could play a role in the common parts of the biosynthesis pathways (i.e. MBC production).

This study has identified the biosynthetic genes responsible for the production of a newly described tambjamine compound, harboured in a surface-associated marine bacterium. Functional genomics-based studies of bioactives from bacteria derived from targeted environmental habitats constitute a powerful tool for identifying novel antimicrobials. Specifically, the knowledge gained here concerning the genes involved in tambjamine production should allow for further exploration into the microbial origin and the distribution of tambjamines in the marine environment. The successful recombinant production of tambjamines in *E. coli* will also benefit the further biotechnological exploitation of this potent group of marine derived bioactives.

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