



**HAL**  
open science

# The B-12-Radical SAM enzyme PoyC catalyzes valine C-beta-Methylation during Polytheonamide Biosynthesis

Auberie Parent, Alain Guillot, Alhosna Benjdia, Gwladys Chartier, Jerome Leprince, Olivier Berteau

## ► To cite this version:

Auberie Parent, Alain Guillot, Alhosna Benjdia, Gwladys Chartier, Jerome Leprince, et al.. The B-12-Radical SAM enzyme PoyC catalyzes valine C-beta-Methylation during Polytheonamide Biosynthesis. *Journal of the American Chemical Society*, 2016, 138 (48), pp.15515-15518. 10.1021/jacs.6b06697 . hal-01605179

**HAL Id: hal-01605179**

**<https://hal.science/hal-01605179>**

Submitted on 13 Dec 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# The B<sub>12</sub>-Radical SAM Enzyme PoyC Catalyzes Valine C<sub>β</sub>-Methylation during Polytheonamide Biosynthesis

Aubérie Parent,<sup>†</sup> Alain Guillot,<sup>†</sup> Alhosna Benjdia,<sup>†</sup> Gwladys Chartier,<sup>†</sup> Jérôme Leprince,<sup>‡</sup> and Olivier Bertheau<sup>\*,†,§</sup>

<sup>†</sup>Micalis Institute, ChemSyBio, INRA, AgroParisTech, Université Paris-Saclay, F-78350 Jouy-en-Josas, France

<sup>‡</sup>INSERM U982, Université Rouen-Normandie, F-76821 Mont-Saint-Aignan, France

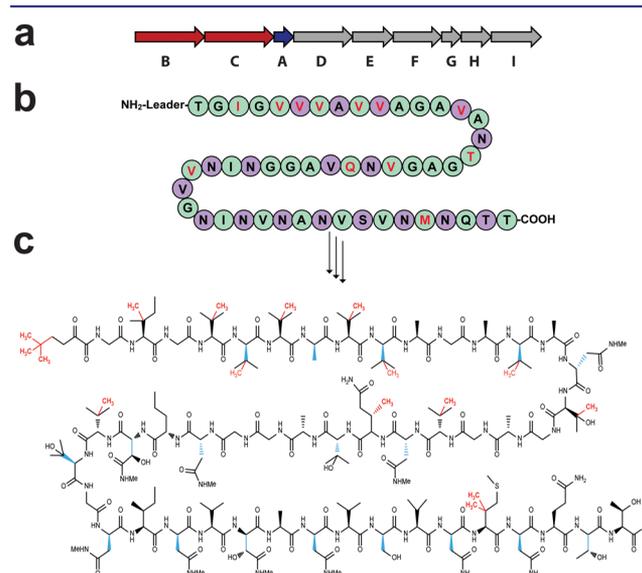
## Supporting Information

**ABSTRACT:** Genomic and metagenomic investigations have recently led to the delineation of a novel class of natural products called ribosomally synthesized and post-translationally modified peptides (RiPPs). RiPPs are ubiquitous among living organisms and include pharmaceutically relevant compounds such as antibiotics and toxins. A prominent example is polytheonamide A, which exhibits numerous post-translational modifications, some of which were unknown in ribosomal peptides until recently. Among these post-translational modifications, C-methylations have been proposed to be catalyzed by two putative radical S-adenosylmethionine (rSAM) enzymes, PoyB and PoyC. Here we report the *in vitro* activity of PoyC, the first B<sub>12</sub>-dependent rSAM enzyme catalyzing peptide C<sub>β</sub>-methylation. We show that PoyC catalyzes the formation of S-adenosylhomocysteine and 5'-deoxyadenosine and the transfer of a methyl group to L-valine residue. In addition, we demonstrate for the first time that B<sub>12</sub>-rSAM enzymes have a tightly bound MeCbl cofactor that during catalysis transfers a methyl group originating from S-adenosyl-L-methionine. Collectively, our results shed new light on polytheonamide biosynthesis and the large and emerging family of B<sub>12</sub>-rSAM enzymes.

B<sub>12</sub>-dependent radical S-adenosylmethionine (B<sub>12</sub>-rSAM) enzymes<sup>1,2</sup> constitute one of the largest groups of enzymes within the rSAM enzyme superfamily, which contains more than 140 000 members.<sup>3,4</sup> They have been identified in the biosynthetic pathways of many natural products, including several families of ribosomally synthesized and post-translationally modified peptides (RiPPs) such as thiostrepton,<sup>1,2,5</sup> polytheonamides,<sup>6</sup> and bottromycins.<sup>7</sup> B<sub>12</sub>-rSAM enzymes are also involved in the biosynthesis of a wide range of antibiotics produced by Actinomycetes: carbapenem,<sup>8</sup> chondrochloren,<sup>9</sup> clorobiocin,<sup>10</sup> fortimicin,<sup>11</sup> fosfomicin,<sup>12</sup> gentamicin,<sup>13</sup> mitomycin,<sup>14</sup> moenomycin,<sup>15</sup> novobiocin,<sup>16</sup> pactamycin,<sup>17</sup> and L-phosphinothricin.<sup>18</sup> However, their function often remains controversial, and the first *in vitro* studies have been reported only recently.<sup>2,18</sup>

B<sub>12</sub>-rSAM enzymes have been proposed to catalyze chemically challenging C- and P-methyl transfer reactions. Nevertheless, our understanding of their functions and catalytic mechanisms remains very limited. In an effort to gain insights into the unique chemistry of these enzymes and the biosynthesis of poly-

theonamides, we undertook a study of PoyB and PoyC, the two putative B<sub>12</sub>-rSAM enzymes encoded in the *poy* operon (Figure 1a).<sup>6</sup>



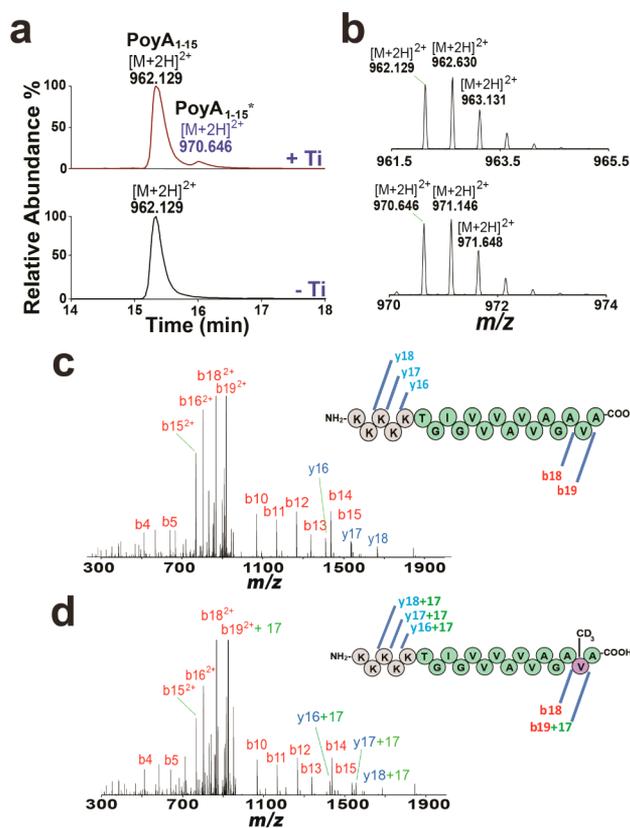
**Figure 1.** (a) Biosynthetic gene cluster of polytheonamide A. The two predicted B<sub>12</sub>-rSAM enzymes PoyB and PoyC (in red) and the precursor peptide PoyA (in blue) are indicated. (b) Sequence of PoyA showing the core peptide. Residues in red indicate C-methylated residues, while purple circles denote epimerized residues. (c) Structure of polytheonamide A. D-Configured residues are shown in blue, and methyl groups proposed to be inserted by PoyB and/or PoyC are shown in red.

Polytheonamides are the founding members of the so-called proteusins. They have been shown to contain 48 post-translational modifications, with 35 predicted to be catalyzed by three rSAM enzymes: PoyB, PoyC, and PoyD (Figure 1b,c). By an *in vivo* approach, it has been shown that PoyD is a novel type of rSAM enzyme generating D-configured amino acid residues.<sup>6</sup> Although no experimental evidence was available, PoyB and PoyC were predicted to be responsible for the 13 C<sub>β</sub>-methylations and the formation of the unique N-terminal *tert*-butyl group (Figure 1c). Interestingly, recent characterization of the bottromycin biosynthetic cluster led to the identification of

Received: June 29, 2016

Published: November 29, 2016

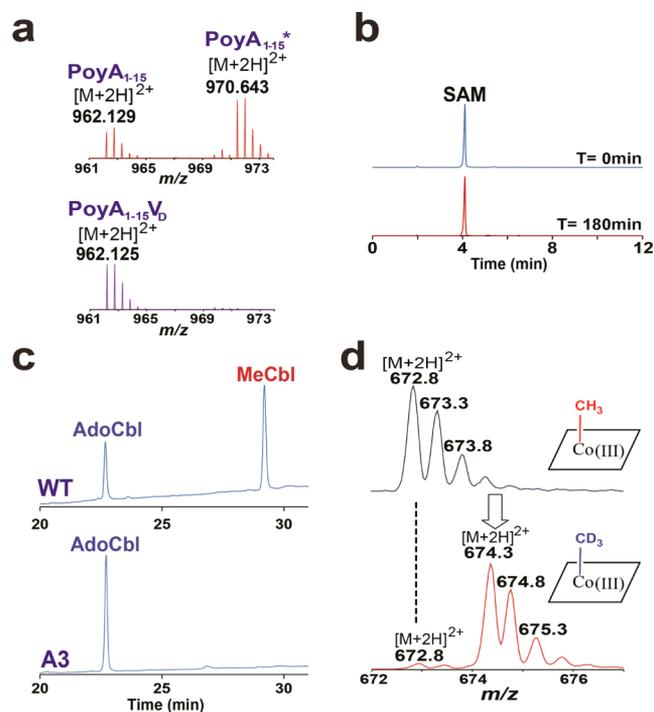




**Figure 3.** (a) LC–MS analysis of PoyA<sub>1–15</sub> incubated with PoyC and CD<sub>3</sub>-SAM under anaerobic conditions in the presence (+Ti) or absence (–Ti) of titanium citrate. See the SI for the experimental conditions. (b) High-resolution LC–MS analyses of PoyA<sub>1–15</sub> (upper trace) and PoyA<sub>1–15</sub>\* (lower trace). (c, d) High-resolution LC–MS/MS analyses of (c) PoyA<sub>1–15</sub> and (d) PoyA<sub>1–15</sub>\*. See Supplementary Figure 2 for the full spectra.

LC–MS/MS analysis of the modified PoyA<sub>1–15</sub>\* peptide revealed the positioning of the methyl group on the penultimate amino acid, L-Val<sub>14</sub> (Figure 3c,d, Supplementary Figure 2, and Supplementary Tables 1 and 2). Interestingly, Val<sub>14</sub> is one of the few C-methylated and epimerized residues found in native polytheonamide A (Figure 1b). Previous studies have shown that *in vivo*, heterologous coexpression of PoyA with PoyD<sup>6</sup> leads to epimerization of amino acid residues, suggesting that epimerization is the first step in polytheonamide A biosynthesis. However, since *E. coli* is unable to produce *de novo* cobalamin, coexpressions of PoyA with PoyB or PoyC were inconclusive. To test whether PoyC acts prior to or after epimerization, we synthesized a peptide containing one D-Val at position 14 (PoyA<sub>1–15</sub>V<sub>D</sub>). As shown, introduction of one D-Val led to methyl transfer inhibition (Figure 4a). This result further supports the hypothesis that PoyC catalyzes methyl transfer only on L-amino acids. Consistent with this hypothesis, most of the C-methylated residues are L-configured in polytheonamide A (Figure 1b).

PoyC possesses a predicted unusual rSAM motif: Cx7Cx2C. To probe its function, we generated the corresponding triple variant Ax7Ax2A (Supplementary Figure 3). As expected, this mutant was unable to produce 5'-dA and to transfer a methyl group to the substrate (Figure 4b). However, contrary to TsrM,<sup>1,2</sup> this mutant was also impaired for SAH production, suggesting different functions for the [4Fe–4S] center in these two B<sub>12</sub>-rSAM enzymes.



**Figure 4.** (a) LC–MS analyses of PoyA<sub>1–15</sub> (upper trace) and PoyA<sub>1–15</sub>V<sub>D</sub> (lower trace) after incubation in the presence of PoyC. See the SI for the experimental conditions. (b) SAM cleavage assay performed with the PoyC Ax7Ax2A mutant (UV detection at 257 nm). Samples were assayed at  $T = 0$  min (upper trace) and after 180 min of incubation with the mutant enzyme (lower trace). See the SI for the experimental conditions. (c) HPLC analysis of the cobalamin derivatives present in PoyC (WT) and the Ax7Ax2A mutant (A3) (UV detection at 278 nm). (d) LC–MS analyses of MeCbl present in PoyC at  $T = 0$  h (upper trace) and after 1 h of incubation with its peptide substrate, Ti (10 mM) and CD<sub>3</sub>-SAM (2 mM) (lower trace).

Because PoyC is the first B<sub>12</sub>-rSAM enzyme isolated natively loaded with a B<sub>12</sub> cofactor (i.e., without addition of cobalamin derivatives during enzyme refolding, purification or reaction), we aimed to characterize its B<sub>12</sub> content. PoyC, carefully handled in the dark, was found to contain essentially two B<sub>12</sub> derivatives identified as AdoCbl and MeCbl by their HPLC profiles (Figure 4c and Supplementary Figure 4), their UV–vis spectra and MS analyses (Supplementary Figure 5). PoyC proved to contain  $0.27 \pm 0.08$  mol of MeCbl and  $0.07 \pm 0.02$  mol of AdoCbl per polypeptide chain.

The apparent nonselectivity of PoyC for the upper axial ligand of cobalamin was surprising since the methionine synthase from *E. coli* was purified as a mixture of forms containing hydroxo- or methylcobalamin<sup>27</sup> while the “corrinoid iron–sulfur protein” was isolated as a cob(II)alamin complex.<sup>28,29</sup> It is well-known that when supplied with cobalamin derivatives, *E. coli* produces large amounts of AdoCbl,<sup>30</sup> which was likely incorporated during the heterologous expression of PoyC. Interestingly, the Ax7Ax2A mutant also contained AdoCbl, but we did not detect MeCbl (Figure 4c). This result suggests a critical role of the [4Fe–4S] center in cobalamin methylation. It is also consistent with the fact that the Ax7Ax2A mutant is unable to produce SAH and thus to transfer the methyl group from SAM to cobalamin.

As shown, the AdoCbl/MeCbl content of the enzyme did not change during the reaction (Supplementary Figure 6). However, MeCbl was almost entirely converted into CD<sub>3</sub>-Cbl during the

reaction (Figure 4d), establishing that the enzyme-bound cobalamin is recycled from SAM during catalysis.

Collectively, our results demonstrate that PoyC is a B<sub>12</sub>-rSAM enzyme with an unusual Cx7Cx2C radical SAM motif identified only in a putative rSAM enzyme<sup>31</sup> and the predicted bottromycin methyltransferases.<sup>7</sup> We purified PoyC with two bound alkylcobalamins: AdoCbl and MeCbl. On the basis of the UV–vis spectrum, it appears that these cobalamins have a base-off coordination, similar to what has recently been proposed for TsrM.<sup>20</sup> Using Ti as the reducing system, we have also demonstrated that PoyC is a peptide C-methyltransferase catalyzing the reductive cleavage of SAM. Ti has been extensively used for the reactivation of methionine synthase<sup>26</sup> and recently with a B<sub>12</sub>-dependent P-methyltransferase.<sup>24</sup> However, in this latter case, no 5'-dA or SAH was reported.

In agreement with our results, it is reasonable to assume that PoyC catalyzes the reductive cleavage of SAM into the 5'-dA radical. This radical species likely abstracts a C<sub>β</sub>-H atom,<sup>32</sup> generating a carbon-centered radical intermediate. Currently, how the methyl group is attached and what are the physiological oxidation states of cobalamin remain open questions. The homolytic cleavage of MeCbl and the formation of a methyl radical intermediate, as elegantly reported in methyl-coenzyme M reductase,<sup>33</sup> would provide a reasonable solution to attach the methyl group to the carbon-centered radical. Cob(II)alamin, after reduction to cob(I)alamin, could react with SAM to regenerate MeCbl. We have previously shown that TsrM catalyzes methyl transfer to sp<sup>2</sup> carbon atoms<sup>2</sup> and rigorously demonstrated that TsrM, contrary to PoyC, does not catalyze the reductive cleavage of SAM or abstracts a substrate H atom.<sup>1,2</sup> It thus appears that, depending on the hybridization of the carbon atom (sp<sup>2</sup> vs sp<sup>3</sup>), B<sub>12</sub>-rSAM enzymes have evolved different mechanisms requiring or not requiring the formation of 5'-dA radical.

Finally, our data show that PoyC catalyzes methyl transfer only on L-amino acid residues. This result indicates that methylation occurs prior epimerization and suggests that PoyB is likely responsible for the synthesis of the N-terminal *tert*-butyl group in polytheonamide A, although other roles cannot be ruled out yet.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06697.

Experimental procedures and supplementary figures (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

\*Olivier.Berteau@inra.fr

### ORCID

Olivier Berteau: 0000-0002-3434-5168

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was supported by the European Research Council (Consolidator Grant 617053 to O.B.). High-resolution MS analyses were performed on the INRA PAPPSO proteomics platform.

## ■ REFERENCES

- (1) Benjdia, A.; Pierre, S.; Gherasim, C.; Guillot, A.; Carmona, M.; Amara, P.; Banerjee, R.; Berteau, O. *Nat. Commun.* **2015**, *6*, 8377.
- (2) Pierre, S.; Guillot, A.; Benjdia, A.; Sandstrom, C.; Langella, P.; Berteau, O. *Nat. Chem. Biol.* **2012**, *8* (12), 957–9.
- (3) Broderick, J. B.; Duffus, B. R.; Duschene, K. S.; Shepard, E. M. *Chem. Rev.* **2014**, *114* (8), 4229–317.
- (4) Benjdia, A.; Berteau, O. *Biochem. Soc. Trans.* **2016**, *44* (1), 109–15.
- (5) Kelly, W. L.; Pan, L.; Li, C. *J. Am. Chem. Soc.* **2009**, *131* (12), 4327–34.
- (6) Freeman, M. F.; Gurgui, C.; Helf, M. J.; Morinaka, B. I.; Uria, A. R.; Oldham, N. J.; Sahl, H. G.; Matsunaga, S.; Piel, J. *Science* **2012**, *338* (6105), 387–90.
- (7) Huo, L.; Rachid, S.; Stadler, M.; Wenzel, S. C.; Muller, R. *Chem. Biol.* **2012**, *19* (10), 1278–87.
- (8) Marous, D. R.; Lloyd, E. P.; Buller, A. R.; Moshos, K. A.; Grove, T. L.; Blaszczyk, A. J.; Booker, S. J.; Townsend, C. A. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (33), 10354–8.
- (9) Rachid, S.; Scharfe, M.; Blocker, H.; Weissman, K. J.; Muller, R. *Chem. Biol.* **2009**, *16* (1), 70–81.
- (10) Westrich, L.; Heide, L.; Li, S. M. *ChemBioChem* **2003**, *4* (8), 768–73.
- (11) Dairi, T.; Ohta, T.; Hashimoto, E.; Hasegawa, M. *Mol. Gen Genet* **1992**, *236* (1), 39–48.
- (12) Woodyer, R. D.; Li, G.; Zhao, H.; van der Donk, W. A. *Chem. Commun. (Cambridge, U. K.)* **2007**, *4*, 359–61.
- (13) Kim, H. J.; McCarty, R. M.; Ogasawara, Y.; Liu, Y. N.; Mansoorabadi, S. O.; Levieux, J.; Liu, H. W. *J. Am. Chem. Soc.* **2013**, *135*, 8093–6.
- (14) Mao, Y.; Varoglu, M.; Sherman, D. H. *Chem. Biol.* **1999**, *6* (4), 251–63.
- (15) Ostash, B.; Walker, S. *Nat. Prod. Rep.* **2010**, *27* (11), 1594–617.
- (16) Steffensky, M.; Muhlenweg, A.; Wang, Z. X.; Li, S. M.; Heide, L. *Antimicrob. Agents Chemother.* **2000**, *44* (5), 1214–22.
- (17) Kudo, F.; Kasama, Y.; Hirayama, T.; Eguchi, T. *J. Antibiot.* **2007**, *60* (8), 492–503.
- (18) Werner, W. J.; Allen, K. D.; Hu, K.; Helms, G. L.; Chen, B. S.; Wang, S. C. *Biochemistry* **2011**, *50* (42), 8986–8.
- (19) Allen, K. D.; Wang, S. C. *Arch. Biochem. Biophys.* **2014**, *543*, 67–73.
- (20) Blaszczyk, A. J.; Silakov, A.; Zhang, B.; Maiocco, S. J.; Lanz, N. D.; Kelly, W. L.; Elliott, S. J.; Krebs, C.; Booker, S. J. *J. Am. Chem. Soc.* **2016**, *138* (10), 3416–26.
- (21) Kim, J.; Gherasim, C.; Banerjee, R. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (38), 14551–4.
- (22) Banerjee, R. V.; Frasca, V.; Ballou, D. P.; Matthews, R. G. *Biochemistry* **1990**, *29* (50), 11101–9.
- (23) Burkhart, B. J.; Hudson, G. A.; Dunbar, K. L.; Mitchell, D. A. *Nat. Chem. Biol.* **2015**, *11* (8), 564–70.
- (24) Allen, K. D.; Wang, S. C. *Biochim. Biophys. Acta, Proteins Proteomics* **2014**, *1844* (12), 2135–2144.
- (25) Matthews, R. G.; Koutmos, M.; Datta, S. *Curr. Opin. Struct. Biol.* **2008**, *18* (6), 658–66.
- (26) Chen, Z.; Chakraborty, S.; Banerjee, R. *J. Biol. Chem.* **1995**, *270* (33), 19246–9.
- (27) Banerjee, R. V.; Johnston, N. L.; Sobeski, J. K.; Datta, P.; Matthews, R. G. *J. Biol. Chem.* **1989**, *264* (23), 13888–95.
- (28) Menon, S.; Ragsdale, S. W. *Biochemistry* **1998**, *37* (16), 5689–98.
- (29) Kung, Y.; Ando, N.; Doukov, T. I.; Blasiak, L. C.; Bender, G.; Seravalli, J.; Ragsdale, S. W.; Drennan, C. L. *Nature* **2012**, *484* (7393), 265–9.
- (30) Escalante-Semerena, J. C.; Warren, M. J. *EcoSal Plus* **2008**, DOI: 10.1128/ecosalplus.3.6.3.8.
- (31) Hiratsuka, T.; Suzuki, H.; Kariya, R.; Seo, T.; Minami, A.; Oikawa, H. *Angew. Chem., Int. Ed.* **2014**, *53* (21), 5423–6.
- (32) Benjdia, A.; Leprince, J.; Sandstrom, C.; Vaudry, H.; Berteau, O. *J. Am. Chem. Soc.* **2009**, *131* (24), 8348–9.
- (33) Wongnate, T.; Sliwa, D.; Ginovska, B.; Smith, D.; Wolf, M. W.; Lehnert, N.; Raugei, S.; Ragsdale, S. W. *Science* **2016**, *352* (6288), 953–8.