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## Reactions of the Three AmpD Enzymes of *Pseudomonas aeruginosa*

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### Abstract

A group of Gram-negative bacteria, including the problematic pathogen *Pseudomonas aeruginosa*, has linked the steps in cell-wall recycling with the ability to manifest resistance to  $\beta$ -lactam antibiotics. A key step at the crossroads of the two events is performed by the protease AmpD, which hydrolyzes the peptide in the metabolite that influences these events. In contrast to other organisms that harbor this elaborate system, the genomic sequences of *P. aeruginosa* reveal it to have three paralogous genes for this protease, designated as *ampD*, *ampDh2* and *ampDh3*. The recombinant gene products were purified to homogeneity and their functions were assessed by the use of synthetic samples of three bacterial metabolites in cell-wall recycling and of three surrogates of cell-wall peptidoglycan. The results unequivocally identify AmpD as the *bona fide* recycling enzyme and AmpDh2 and AmpDh3 as enzymes involved in turnover of the bacterial cell wall itself. These findings define for the first time the events mediated by these three enzymes that lead to turnover of a key cell-wall recycling metabolite as well as the cell wall itself in its maturation.

Members of *Enterobacteriaceae* and *Pseudomonas aeruginosa* have evolved an elaborate sequence of events that recycles more than 50% of the bacterial cell wall during normal growth for reasons that are not fully understood.<sup>1–5</sup> The recycling also takes place when damage to the cell wall occurs. As damage to cell wall is also inflicted by exposure of bacteria to  $\beta$ -lactam antibiotics, these organisms have evolved a link between the biochemical steps of recycling and repair to unleashing of an inducible antibiotic-resistance mechanism involving the AmpC  $\beta$ -lactamase (Fig. 1).<sup>1,5–8</sup>

Bacterial cell-wall recycling commences by degradation of the peptidoglycan, the major constituent of the cell wall, by the family of lytic transglycosylases.<sup>3,4,9,10</sup> Whereas these enzymes turn over their polymeric substrates with some variations on the non-hydrolytic reaction that generates the fragmentation products, the major end product is *N*-acetyl- $\beta$ -D-glucosamine-(1 $\rightarrow$ 4)-1,6-anhydro-*N*-acetyl- $\beta$ -D-muramyl-peptide (**1**), with the full-length peptide being L-Ala-D- $\gamma$ -Glu-*meso*-DAP-D-Ala-D-Ala (DAP stands for diaminopimelate).<sup>9</sup>

Metabolite **1** is internalized by the permease AmpG. Once in the cytoplasm, the glycosidase NagZ converts compound **1** to **2**, which in turn serves as the substrate for the protease AmpD, which removes the peptide stem from the saccharide. The product of this reaction, compound **3**, enters a sequence of biochemical events that synthesizes the building unit

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Supporting Information. Experimental procedures for syntheses of the new compounds, cloning, enzyme purification, kinetics, MS analyses of products and copies of NMR spectra of the new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Lipid II, which is transported to the periplasmic side of the inner membrane to be used in assembly of the nascent peptidoglycan (Fig. 1). As a branching point in these events, metabolite **2** stimulates gene transcription to result in the production of an antibiotic-resistance enzyme, the AmpC  $\beta$ -lactamase (Fig. 1). Hence, the action of AmpD, at the crossroads of these events, shuts down the production of the AmpC  $\beta$ -lactamase and commits to recycling of the cell wall.<sup>3,5,11</sup>

In contrast to other organisms that possess this system, analyses of the genomic sequences of *P. aeruginosa* have led to the annotation of three paralogous genes for the protease AmpD. These are designated as AmpD, AmpDh2 and AmpDh3.<sup>12</sup> AmpDh2 has a signal peptide that targets it to the periplasm and an anchor that is believed to insert it into the outer membrane.<sup>13</sup> On the other hand, AmpD is believed to be cytoplasmic and the cellular location of AmpDh3 is currently unknown.<sup>13</sup> Abrogation of the respective activities for these enzymes by mutational inactivation or by gene ablation led to a stepwise upregulation of antibiotic resistance, with the full effect achieved only by the loss of all three enzyme activities at the whole-organismal level.<sup>12–14</sup> Interestingly, inactivation of *ampD* is not sufficient to affect fitness or virulence of *P. aeruginosa* in murine models of infection, but double or triple mutants with losses of additional activities of the other two enzymes (AmpDh2 and AmpDh3) would appear to be defective in both.<sup>13</sup>

Notwithstanding the careful analyses of the mutational effects at the whole-organismal level, the activities of these three AmpD enzymes of *P. aeruginosa* have not been studied with suitable substrates by enzymological analysis. This is largely due to the lack of availability of these substrates. We report herein our cloning of the genes *ampD*, *ampDh2* and *ampDh3* from the strain *P. aeruginosa* PA01. The respective proteins (AmpD, AmpDh2, and AmpDh3) were expressed and purified to homogeneity. We synthesized compounds **4–9** (Chart 1) by the methodology that we have reported earlier.<sup>15–17</sup> Compounds **5** and **8** had not been made previously and were prepared for the first time for this study (described below), along with the other four known compounds.<sup>16,17</sup> As indicated in Fig. 1, 1,6-anhydromuramyl derivatives **2** are *bona fide* substrates for AmpD. The variation among compounds **2** is in the length of the peptide stem, which is traced back to what is found in the cell wall. This is a uniquely bacterial peptide, whose full-length sequence is L-Ala-D- $\gamma$ -Glu-*meso*-DAP-D-Ala-D-Ala. Maturation of cell wall generates variants of the peptide stem with loss of amino acids from the C-terminal end.<sup>3,18,19</sup> As such, the tripeptide and tetrapeptide stems are more common, and the pentapeptide, which is biosynthetically introduced into the nascent peptidoglycan, is less so.<sup>3,18</sup> We have synthesized all three versions (**4–6**). We have used compounds **7–9** as mimetics of the standard *N*-acetylglucosamine-based muropeptides from the cell wall (Chart 1). These compounds would allow detection of turnover of standard cell-wall peptidoglycan (which exists only in the periplasm), as opposed to that of the 1,6-anhydromuramyl derivatives that are formed by the action of lytic transglycosylases.

Syntheses of the tetrapeptide-containing derivatives (**5** and **8**) are shown in Scheme 1. The key tetrapeptide intermediate **13** was prepared by the same methodology developed for the synthesis of pentapeptide by our laboratory.<sup>16</sup> The synthesis started with the acetamide **10**, which was prepared in 13 steps according to the method of Hernández and Martín.<sup>20</sup> The carboxylate moiety in compound **10** was activated by NHS/EDCI and the resulting NHS ester was treated with D-alanyl benzyl ester to give compound **11**. The three-step transformation of the acetamide functionality to benzyl ester went smoothly using these conditions, which were used initially for the synthesis of pentapeptide.<sup>16</sup>

The Boc group in **12**, was removed in the presence of trifluoroacetic acid and the resultant product was readily coupled to Boc-Ala-D-Glu(ONHS)-OBn to give the key tetrapeptide **13**.

The Boc group in **13** was removed and the resultant amine **14** was allowed to react with either 1,6-anhydromuramic NHS-ester **15**<sup>17</sup> or muramic NHS-ester **16**.<sup>15,16</sup> Global deprotection of **17** or **18** by catalytic hydrogenation (**18** required acid treatment prior to hydrogenation) gave the final anhydromuramyl tetrapeptide **5** or muramyl tetrapeptide **8**, respectively.

With the availability of all three enzymes and the six compounds as potential substrates for these enzymes, we performed kinetics. The kinetics were HPLC-based and we monitored quantitatively the consumption of the substrate and the formation of products. The products were individually analyzed by mass spectrometry as to delineate the nature of the individual reactions. This type of analysis is exemplified by turnover of compound **4** by the protease AmpD, as documented in Figure 2. The peak corresponding to **4** diminished as the reaction proceeded, with two additional peaks appearing, for which the chemical structures were assigned by mass spectrometry as compounds **3** and **19**. Hence, the nature of the enzymatic reaction of AmpD with this compound is hydrolysis of the amide bond to the lactyl moiety. Quantification of the turnover with the use of an internal standard in each case was performed and the kinetic parameters were determined (Table 1). The very same type of analysis was performed for the reactions of each substrate with each enzyme, revealing that we were monitoring hydrolysis of the amide bond at the lactyl moiety in every case. The results are given in Table 1.

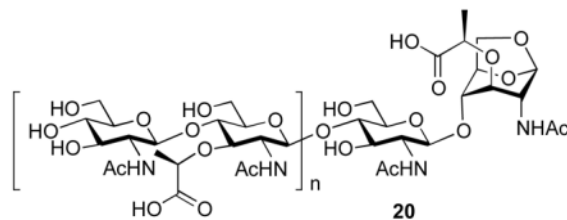
The important observation from these data is that AmpD is the true protease for the recycling process in *P. aeruginosa*, as its function is exclusive for turnover of 1,6-anhydromuramyl variants **4–6**. This enzyme does not turn over variants **7–9**, which are mimetics of the standard peptidoglycan. The AmpD enzyme has evolved for turnover of 1,6-anhydromuramyl compounds, akin to the reaction that we have documented earlier for the enzyme from *Citrobacter freundii*.<sup>17</sup>

On the other hand, AmpDh2 and AmpDh3 exhibit activities with both types of substrates. However, the activities of AmpDh2 and AmpDh3 with the 1,6-anhydromuramyl compounds **4–6** are a mere 6% and 12%, respectively, of the total activity (as assessed by the  $k_{\text{cat}}/K_{\text{m}}$  values). For these two enzymes the overwhelming activity is documented for derivatives **7–9** as substrates. Hence, it is clear that AmpDh2 and AmpDh3 have evolved as enzymes for processing of the peptidoglycan components of the cell wall in the periplasmic space. The mere 6–12% hydrolytic activity with the 1,6-anhydromuramyl compounds **4–6** that we measure for these two enzymes are nonetheless real, a subject to which we return below. However, this marginal activity is likely to be adventitious. Since these enzymes clearly prefer the forms of the peptidoglycan found in the cell wall, they are likely counterparts to the cell-wall amidases such as AmiA, B, C and D, which have been known in *Escherichia coli*.<sup>3</sup>

The AmpD enzyme, the *bona fide* recycling protease, reaches saturation ( $K_{\text{m}}$ ) at low millimolar range with its substrates (Table 1), indicating that the intracellular concentrations of metabolites **4–6** during active cell-wall recycling must be in that range. The rather high concentration is not uncommon for other important bacterial metabolites as well.<sup>15,21,22</sup> This observation regarding the  $K_{\text{m}}$  of metabolites **4–6** holds true for the other two enzymes as well, although their true function, per our findings, is processing of cell wall in the periplasm. We also note that all three enzymes exhibit modest preferences for the tetrapeptide stems in the substrates ( $k_{\text{cat}}/K_{\text{m}}$  effect). Whereas the importance of this observation is not immediately apparent, we are aware that muropeptides with tetrapeptide stem are among the most abundant components in the peptidoglycan analyses of *E. coli* and *P. aeruginosa*.<sup>3,18</sup> It is the reaction of DD-carboxypeptidases, which generates the tetrapeptide version.<sup>3,15,23,24</sup>

If AmpDh2 and AmpDh3 were to be periplasmic enzymes, as proposed in this report, then they will never come in contact with compounds **4–6**, the cytoplasmic metabolites, notwithstanding their marginal ability to turn them over. This is a curiosity, as we have noted that inactivation of the genes for these two enzymes results in stepwise and incremental increase in antibiotic resistance.<sup>12,13</sup> However, AmpDh2 and AmpDh3 do encounter compound **1** in the periplasm. Could the effect be manifested by AmpDh2 and AmpDh3 removing the peptide from metabolite **1**, at least for a fraction of its total concentration? Indeed, this is the case. Compound **1**, which we synthesized by a reported method,<sup>25</sup> is a poor substrate for both AmpDh2 and AmpDh3:  $k_{\text{cat}} = 0.45 \pm 0.03 \text{ s}^{-1}$ ,  $K_{\text{m}} = 1.0 \pm 0.1 \text{ mM}$  and  $k_{\text{cat}}/K_{\text{m}} = 440 \pm 53 \text{ M}^{-1}\text{s}^{-1}$  for AmpDh2;  $k_{\text{cat}} = 2.2 \pm 0.2 \text{ s}^{-1}$ ,  $K_{\text{m}} = 1.2 \pm 0.3 \text{ mM}$  and  $k_{\text{cat}}/K_{\text{m}} = 1800 \pm 480 \text{ M}^{-1}\text{s}^{-1}$  for AmpDh3. Hence, the primary reaction of AmpDh2 and AmpDh3 is turnover of the normal cell-wall peptidoglycan. However, these two enzymes commence removal of the peptide from a fraction of **1** in the periplasm, prior to the completion of the process by the action of AmpD within the cytoplasm.

That the cell-wall surrogate compounds **7–9** were turned over by AmpDh2 and AmpDh3 was intriguing. However, do these enzymes actually turn over the cell wall itself? To address this issue, we prepared the *P. aeruginosa* sacculus according to a reported method.<sup>9,26</sup> Overnight incubation of the polymeric sacculus with AmpDh2 produced products with oligomeric cell-wall sugars without the peptide stem. Mass spectrometric detection identified  $m/z$  values of  $479^+$ ,  $957^+$ ,  $1435^+$ ,  $957^{2+}$ ,  $1196^{2+}$ ,  $1436^{2+}$ ,  $1117^{3+}$ , and  $1276^{3+}$ , corresponding to the general formula,  $(\text{NAG-NAM})_n\text{-NAG-anhMur}$  (structure **20**,  $n = 0–7$ ). The reaction with AmpDh3 also produced products (**20**), but its quantity was three times less than that with AmpDh2.



In summary, we have provided the first enzymological analyses of reactions of AmpD, AmpDh2 and AmpDh3 from *P. aeruginosa* with unique synthetic substrates (three bacterial metabolites and three structural surrogates for the cell wall). *This analysis identifies AmpD of P. aeruginosa as the cytoplasmic protease for commitment to the cell-wall recycling events and for the reversal of the activation of the antibiotic-resistance pathway.* The enzymes AmpDh2 and AmpDh3 exhibited marginal activities with the 1,6-anhydromurmyl compounds **4–6** as substrates. Their function(s) is turnover of cell wall within the periplasmic space. Nonetheless, the marginal activity in turnover of metabolite **1** with AmpDh2 and AmpDh3 explains the stepwise effect observed in augmenting derepression of AmpC  $\beta$ -lactamase by eliminations of these enzymes one-by-one.<sup>12,13</sup>

## Supplementary Material

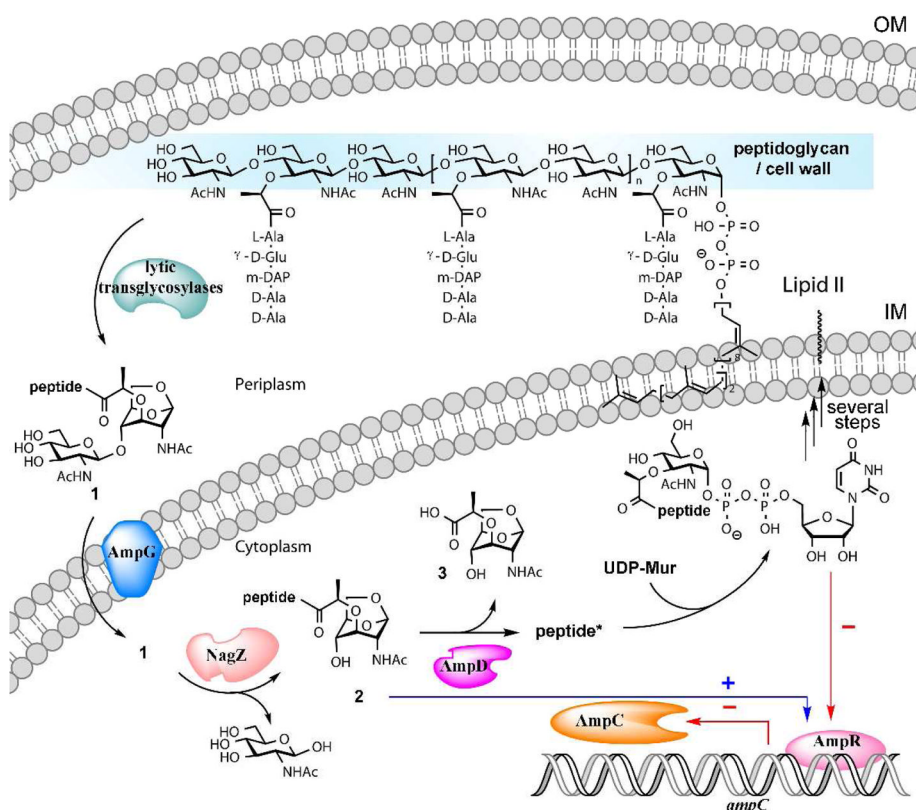
Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

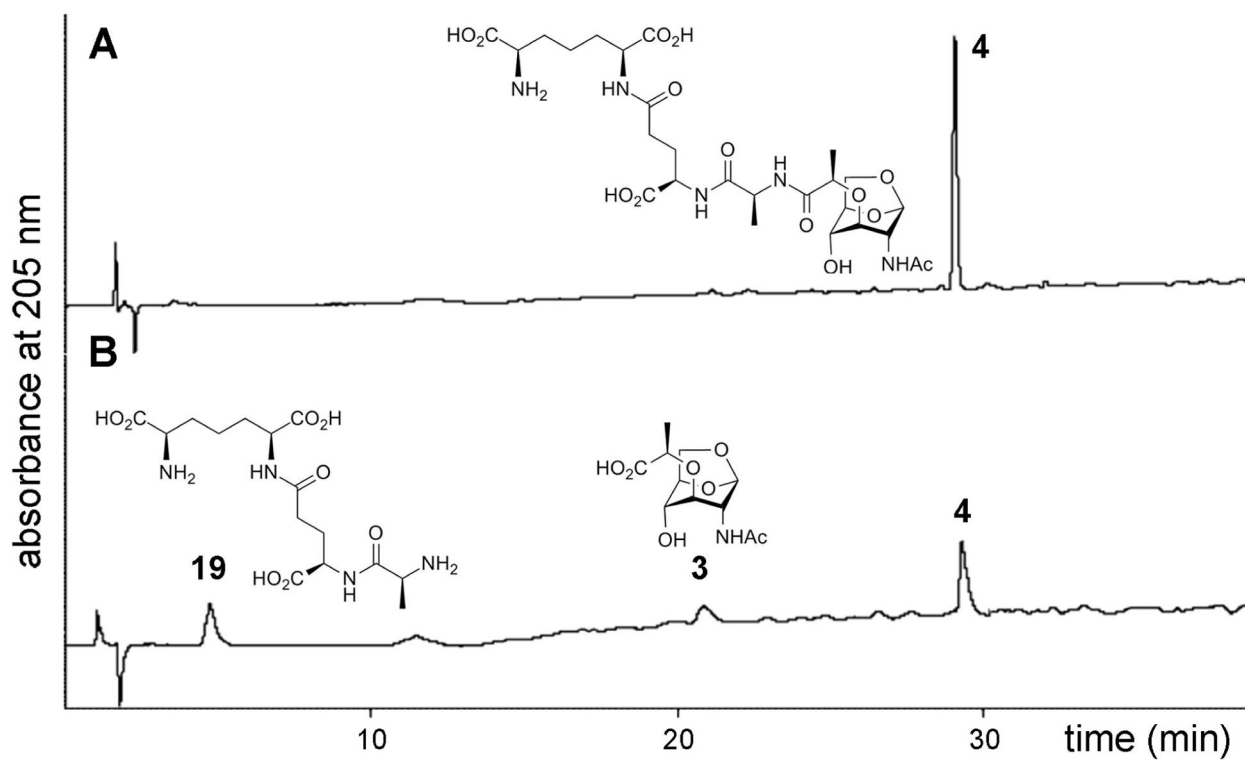
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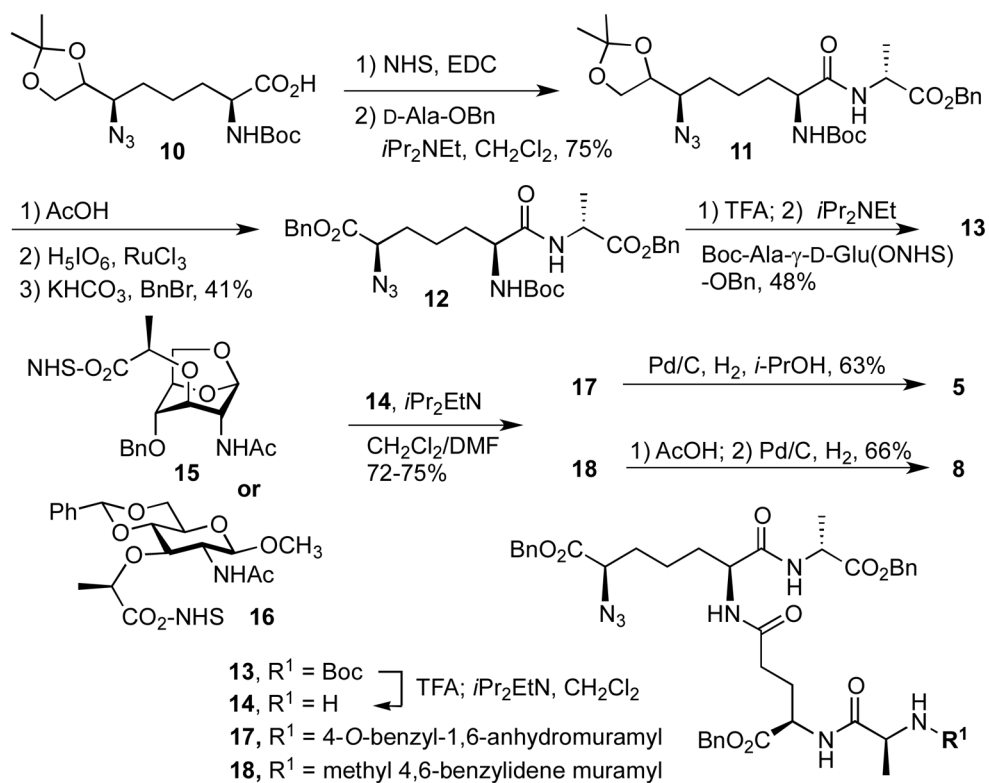
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**Figure 1.** The early events of bacterial cell-wall recycling and their link to antibiotic resistance. Lytic transglycosylases degrade the cell wall in the periplasm, located between the outer membrane (OM) and the inner membrane (IM).

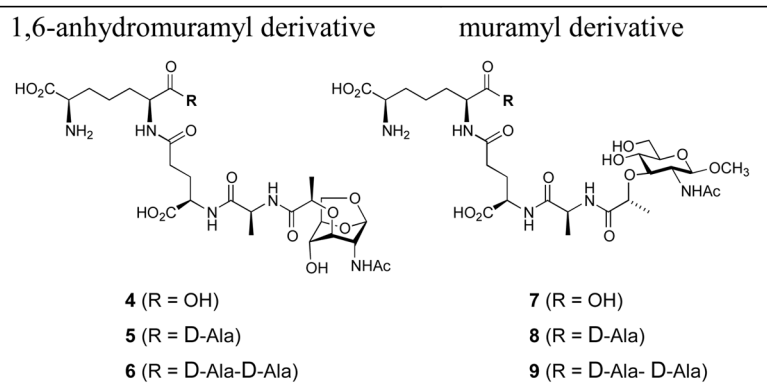


**Figure 2.** The AmpD reaction analysis by HPLC. (A) Reaction mixture containing 100 nM purified AmpD and 3 mM of compound **4** was incubated at 25 °C. (B) Partial transformation identifies compounds **19** and **3** as products, with  $m/z$  values of 391 and 276, respectively.



Scheme 1.





**Chart 1.**  
Chemical structures of substrates for AmpD enzymes used in this study

**Table 1**Steady-state kinetic data for turnover of compounds by AmpD, AmpDh2 and AmpDh3.<sup>a</sup>

Enzyme/Compds	$K_m$ (mM)	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> )	
AmpD	<b>4</b>	2.0 ± 0.1	24.2 ± 3.5	12100 ± 1800
	<b>5</b>	1.2 ± 0.3	39.8 ± 3.1	33200 ± 7500
	<b>6</b>	1.9 ± 0.2	47.5 ± 5.5	25000 ± 4600
	<b>7, 8, 9<sup>b</sup></b>	-	-	-
AmpDh2	<b>4</b>	1.5 ± 0.2	0.20 ± 0.02	130 ± 17
	<b>5</b>	4.6 ± 0.5	20 ± 1.9	4300 ± 790
	<b>6</b>	1.9 ± 0.2	0.18 ± 0.02	95 ± 14
	<b>7</b>	1.5 ± 0.1	51.8 ± 8.9	34500 ± 4300
	<b>8</b>	1.8 ± 0.3	73.6 ± 5.3	40900 ± 6800
	<b>9</b>	1.4 ± 0.2	1.6 ± 0.2	1100 ± 150
AmpDh3	<b>4</b>	3.2 ± 0.5	17.9 ± 1.3	5590 ± 610
	<b>5</b>	4.5 ± 0.6	70.6 ± 6.6	15700 ± 1900
	<b>6</b>	1.4 ± 0.1	20.6 ± 1.9	14700 ± 1600
	<b>7</b>	2.2 ± 0.3	195 ± 24	88600 ± 13600
	<b>8</b>	1.4 ± 0.2	212 ± 19.3	151000 ± 20500
	<b>9</b>	2.4 ± 0.3	85.3 ± 10.2	35500 ± 4800

<sup>a</sup>The activities of all three enzymes decreased in the presence of EDTA, indicating that they are zinc proteases. Kinetic measurements were performed in the presence of 100 μM ZnCl<sub>2</sub> as a supplement for AmpDh2 and AmpDh3.

<sup>b</sup>No measurable activity was detected for AmpD with these compounds.