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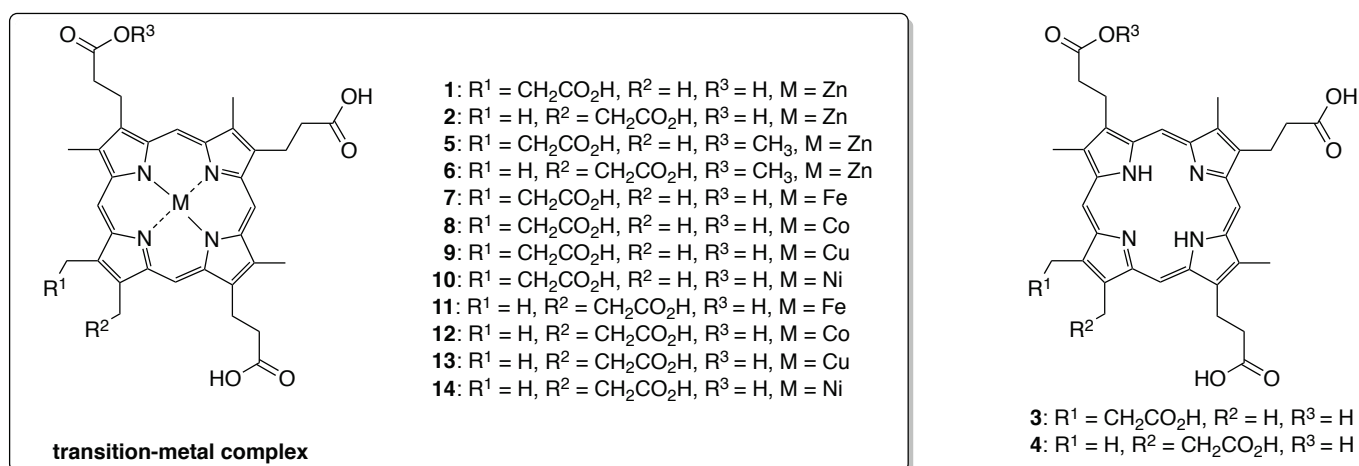
## GROWTH MECHANISM OF UNCULTURED ACTINOBACTERIAL STRAIN *LEUCOBACTER* sp. ASN212 BY ZINC COPROPORPHYRIN

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**Abstract** – We propose HemH as a primary target of Zn coproporphyrin, which stimulates the growth of uncultured *Leucobacter* sp. ASN212, based on the structure-activity relationship for transition-metal complexes of coproporphyrin.

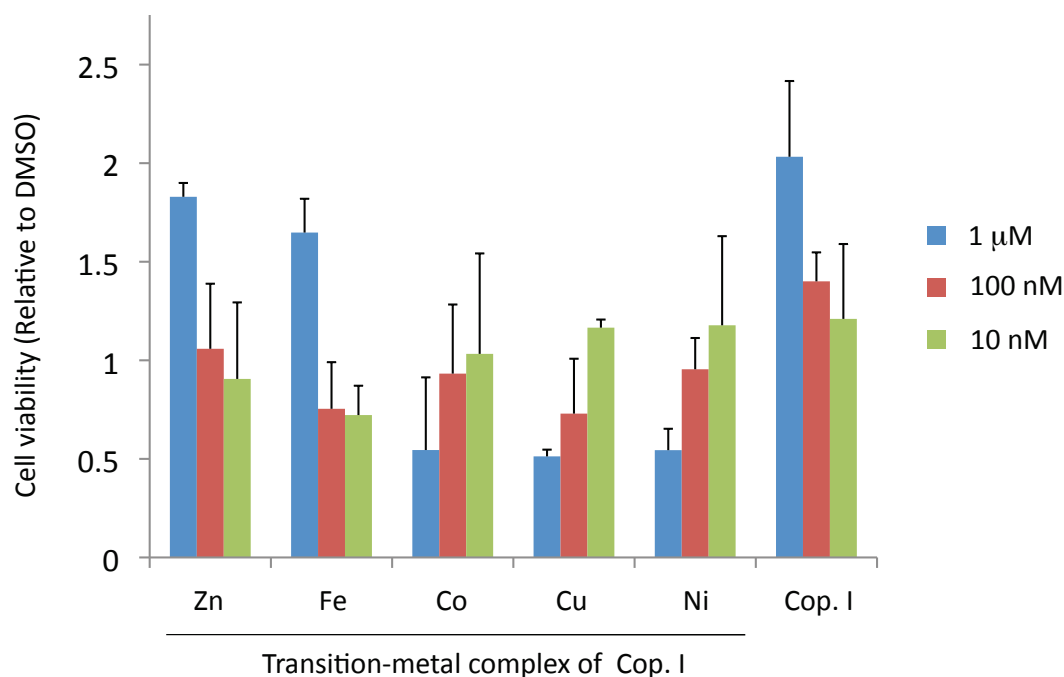
We identified Zn coproporphyrin I (**1**), zincphyrin (**2**), coproporphyrin I (**3**) and III (**4**) together with structurally new zincmethylphyrin I (**5**) and III (**6**) as interphylum growth factors secreted from  $\alpha$ -proteobacterial strain *Sphingopyxis* sp. GF9 for the previously uncultured (here referred to as “uncultured”) actinobacterial strain *Leucobacter* sp. ASN212.<sup>1</sup> This article discusses the structure-activity relationship for a series of transition-metal complexes of **3** and **4**, and proposes a molecular mechanism for how the actinobacterial strain grows by the growth factors across a boundary at phylum level. Figure 1 shows the structures of natural growth factors (**1-6**), and newly synthesized Fe coproporphyrin I (**7**), Co coproporphyrin I (**8**), Cu coproporphyrin I (**9**), Ni coproporphyrin I (**10**), Fe coproporphyrin III (**11**), Co coproporphyrin III (**12**), Cu coproporphyrin III (**13**), and Ni coproporphyrin III (**14**).



**Figure 1.** Structures of coproporphyrin-metal complexes and coproporphyrin I (**3**) and III (**4**)

To unravel the mechanism for growth stimulation of strain ASN212 by growth factors (**1-6**), we conducted a structure-activity relationship study using a series of transition-metal complexes of coproporphyrin (**7-14**). The complexation of transition-metal ion ( $\text{Zn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ , or  $\text{Ni}^{2+}$ ) was investigated by using synthetically available **3**. We first changed the method for complexation of metal ions with **3** concerning the solubility of the complexes. Complexation of  $\text{Zn}^{2+}$  or  $\text{Cu}^{2+}$  with **3** proceeded at room temperature, and direct separation of the metal complex from the reaction mixture by HPLC resulted in successful recovery of **1** or **9**. By contrast, complexation of  $\text{Fe}^{2+}$ ,  $\text{Co}^{2+}$ , or  $\text{Ni}^{2+}$  with **3** did not proceed at room temperature. Fe coproporphyrin I (**7**), Co coproporphyrin I (**8**), and Ni coproporphyrin I (**10**) were synthesized at 100 °C by using **3** with  $\text{Fe}(\text{OAc})_2$ ,  $\text{Co}(\text{OAc})_2$ , and  $\text{Ni}(\text{OAc})_2$ , respectively. Complexation of transition-metal ion with **4** was performed by the same method as described above to give **2**, **11**, **12**, **13**, and **14**.<sup>2</sup>

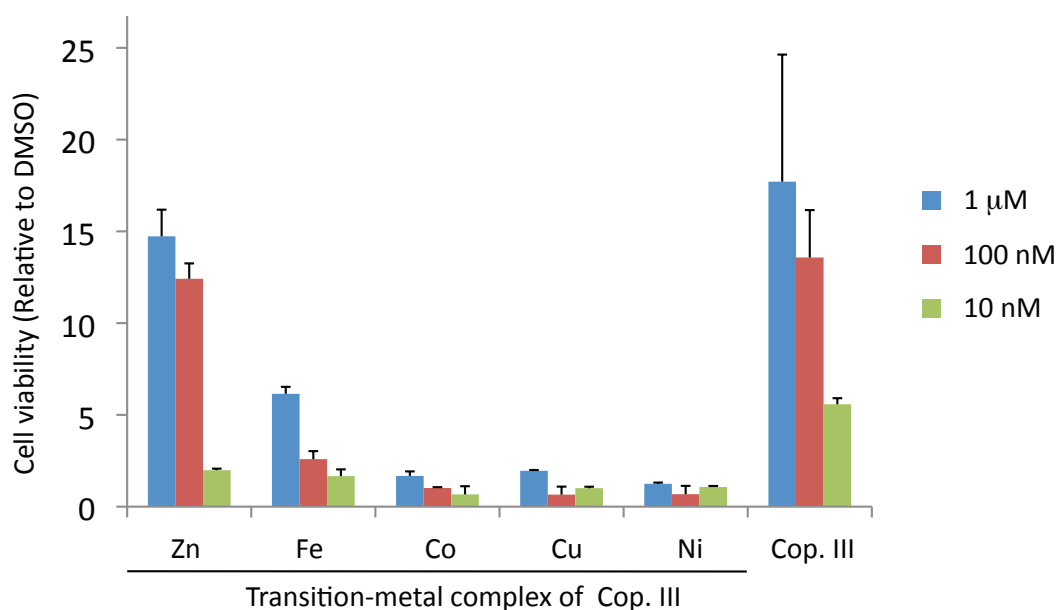
To evaluate growth stimulation by these compounds, we adopted the CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay, which can quantify ATP as luminescence intensity.<sup>3</sup> Growth stimulation of strain ASN212 by Fe coproporphyrin I (**7**) was observed at comparable levels to that induced by the natural growth factor **1** or **3** within the transition-metal complexes of coproporphyrin I as shown in Figure 2.



**Figure 2.** Growth profiles of strain ASN212 by transition-metal complexes of coproporphyrin I. Zn: **1**, Fe: **7**, Co: **8**, Cu: **9**, Ni: **10**, Cop.I: **3**, Cell viability (Relative to DMSO): each luminescence unit was divided by the luminescence unit of vehicle control (0.1% DMSO).

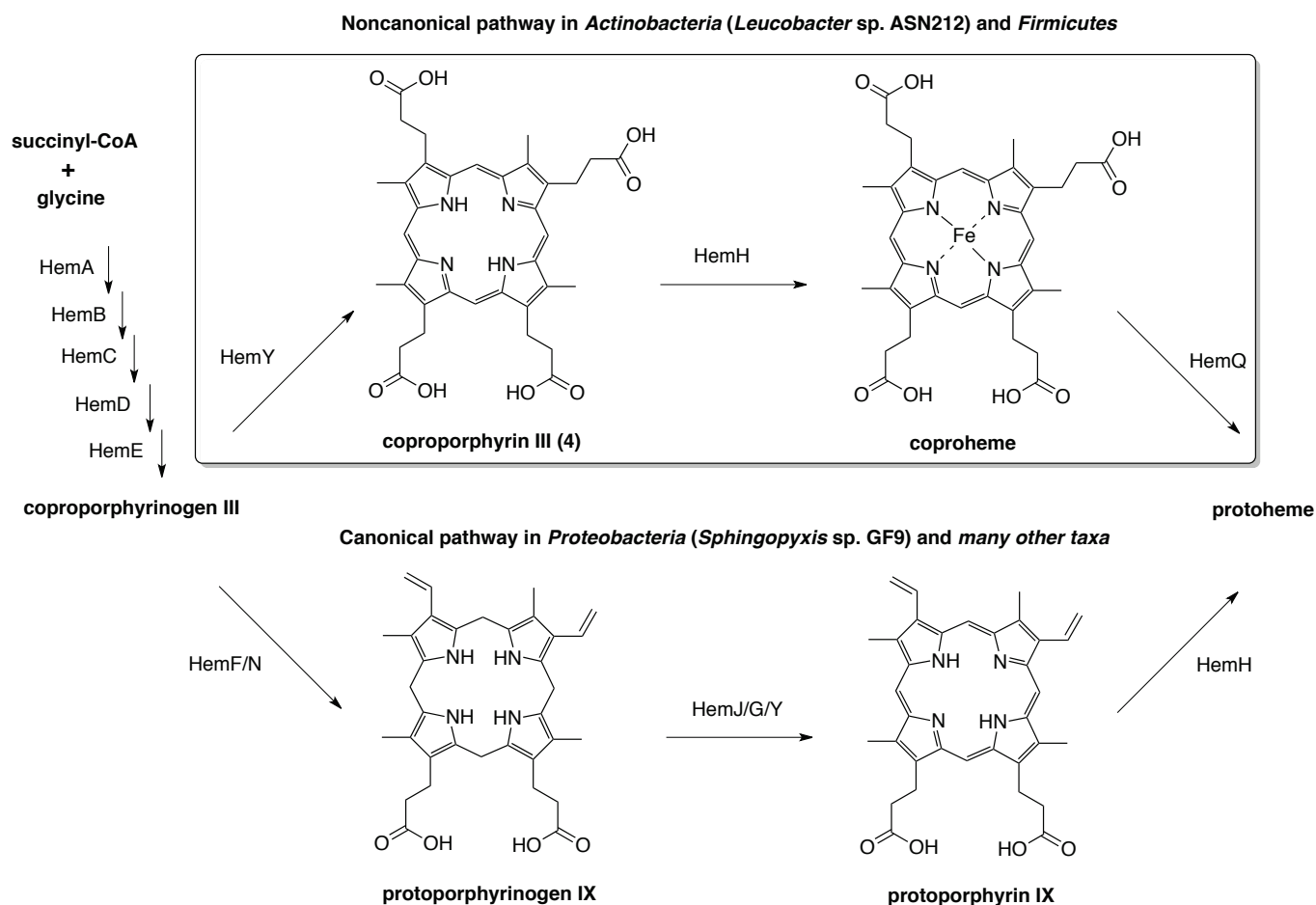
By contrast, Co coproporphyrin I (**8**), Cu coproporphyrin I (**9**), and Ni coproporphyrin I (**10**) did not stimulate the growth significantly. Fe coproporphyrin III (**11**) induced the remarkable growth of strain

ASN212 at comparable levels to zincphyrin (**2**) or coproporphyrin III (**4**), whereas Co coproporphyrin III (**12**), Cu coproporphyrin III (**13**), and Ni coproporphyrin III (**14**) did not show growth activity as in the case of coproporphyrin I series. The activities of **2**, **4** and **11** in coproporphyrin III series were, however, greater than those of **1**, **3**, and **7** in coproporphyrin I series as shown in Figure 3. Relatively weak activity of **11** in coproporphyrin suggests that heme-degrading protein breaks down **11** in a greater or lesser to release  $\text{Fe}^{2+}$  in cytoplasm.<sup>4</sup>



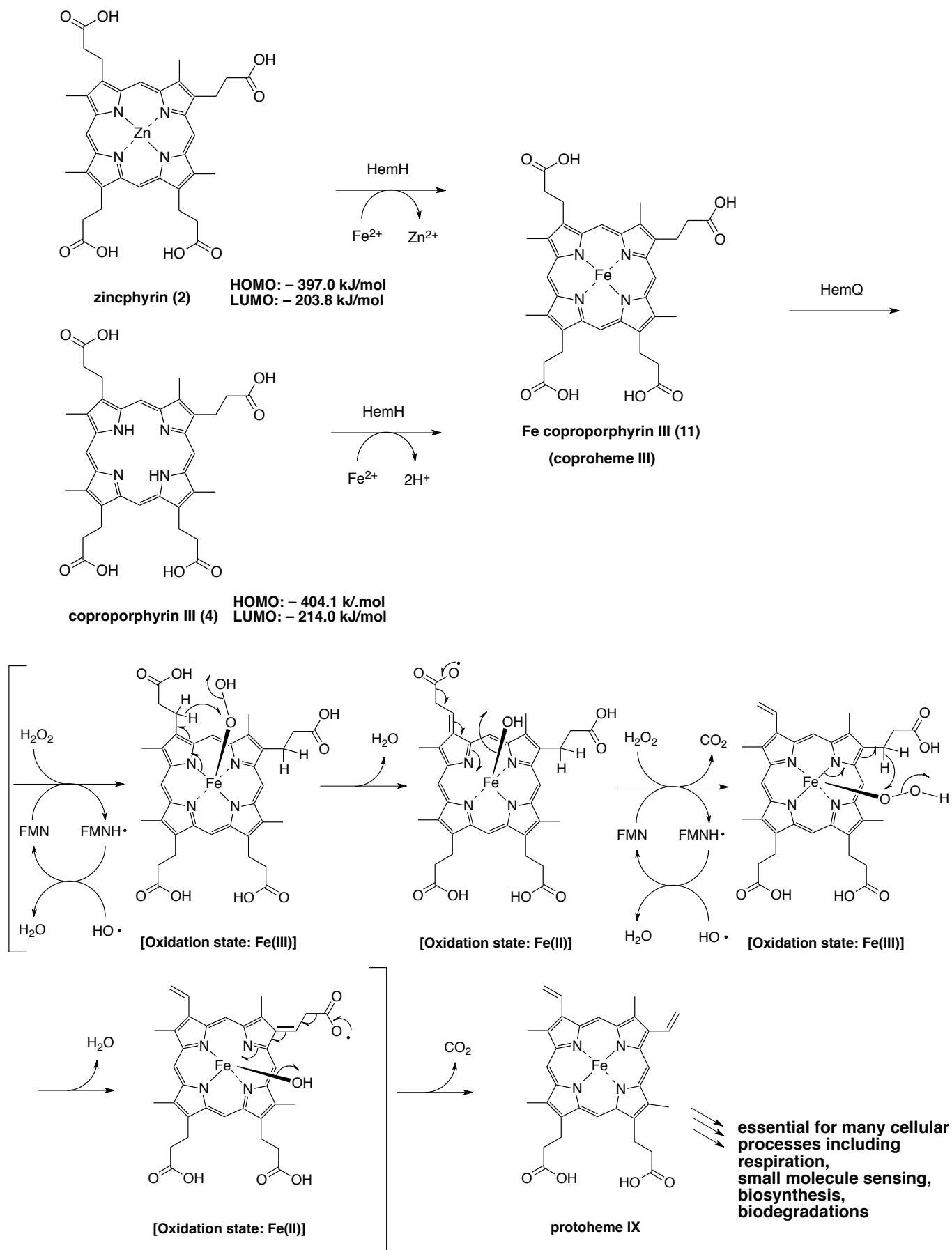
**Figure 3.** Growth profiles of strain ASN212 by transition-metal complexes of coproporphyrin III. Zn: **2**, Fe: **11**, Co: **12**, Cu: **13**, Ni: **14**, Cop.III: **4**, Cell viability (Relative to DMSO): each luminescence unit was divided by the luminescence unit of vehicle control (0.1% DMSO).

Taking into account that  $\text{Zn}^{2+}$  was incorporated in coproporphyrin at room temperature<sup>5</sup> and Fe coproporphyrin **7** and **11** functioned as growth factors for strain ASN212, we propose HemH as a primary target of the natural growth factors (**1-6**) as shown in Figures 4 and 5. Bacteria within the Phyla *Actinobacteria* and *Firmicutes* are unable to synthesize protoporphyrin, instead, these Gram positive bacteria oxidize coproporphyrinogen III to coproporphyrin III (**4**) with HemY, convert **4** to Fe coproporphyrin III (**11**) with HemH, and finally decarboxylate **11** to generate protoheme IX with HemQ via the noncanonical pathway that is different from the canonical pathway in *Proteobacteria* and many other taxa as shown in Figure 4.<sup>6</sup> The function of HemF or HemN in *Sphingopyxis* sp. GF9 and several other proteobacterial strains<sup>1</sup> might be moderately suppressed or the functions of HemA-HemE might be enhanced, because overexpression of *HemA-HemE* with or without *HemY* gene or inhibition of HemN leads to overproduction of coproporphyrin III (**4**).<sup>7,8</sup>



**Figure 4.** Noncanonical heme biosynthesis pathway in *Actinobacteria* including *Leucobacter* sp. ASN212 or *Firmicutes* and canonical pathway in *Proteobacteria* including *Sphingopyxis* sp. GF9 or other taxa

The experiments on complexation of transition-metal ions with **4** suggest that non-enzymatic conversion of **4** to zincphyrin (**2**), the most abundant growth factor in the culture supernatant of strain GF9, easily occur at the culture conditions of strain GF9. Actinobacterial strain ASN212 might carry the defective gene in the committed step or early step of heme biosynthesis, because significant number of heme-synthesizing bacteria lack one or more of the heme-synthesizing enzyme or cause the malfunction of the heme-synthetic machinery.<sup>9</sup> Strain ASN212 can gain ATP only via glycolysis in this situation. The HOMO-LUMO energy gap of Zn coproporphyrin is most resemblance to that of coproporphyrin,<sup>5</sup> thus growth factor **1**, **2**, **3**, **4**, **5**, or **6** can be substrate for HemH after uptake possibly by heme uptake system escaping extreme degradation.<sup>4,10</sup>



**Figure 5.** Plausible mechanism for the growth stimulation of uncultured *Leucobacter* sp. ASN212

In conclusion, Fe coproporphyrin I (**7**) or III (**11**) can be substrate for HemQ, while **1** – **6** can be substrates for HemH as indicated in Figure 5. Co, Cu, or Ni complex of coproporphyrin might rather inhibit the function of HemQ as indicated in Figure 2, whereas slight growth enhancement of strain ASN212 was observed by treating with Co, Cu, or Ni coproporphyrin III in certain concentrations as shown in Figures 3. Dailey et al. reported that inclusion of H<sub>2</sub>O<sub>2</sub> in the reaction of HemQ with coproheme resulted in production of protoheme, whereas the overall coproheme and protoheme present after assay period was less than expected possibly because HemQ-bound heme was degraded by peroxide. Inclusion of flavin mononucleotide (FMN) resulted in conversion of coproheme into protoheme with HemQ, whereas other electron acceptors such as oxygen, NAD, NADP, or FAD had no impact on protoheme production in vitro.<sup>6</sup> A mechanism was proposed from these observations and our findings to explain the oxidative decarboxylation of coproheme by HemQ as indicated in Figure 5. This model explains how zincmethylpyrrole I (**5**) or zincmethylpyrrole III (**6**) functions as growth factor by considering that OH radical released from the first Fe(III)-OOH intermediate degrades the methyl ester to afford MeOH instead of H<sub>2</sub>O and the second Fe(II)-OH intermediate. The model is different from those for catalyses by the HemF-type coproporphyrinogen oxidase that use molecular oxygen as an electron acceptor,<sup>11</sup> the oxygen-independent HemN-type coproporphyrinogen oxidase,<sup>12</sup> and AhbD-type coproheme oxidase within the radical SAM enzyme superfamily.<sup>13</sup>

Although the model should be verified by further investigations, the concept presented in this work could shed some more light on the drug discovery efforts by utilizing uncultured *Actinobacteria*. Given high rates of antimicrobial resistance among *Actinobacteria* and *Firmicutes* and the observation that knock-out of *hemQ* in *Staphylococcus aureus* resulted in the small colony variant phenotype,<sup>14</sup> the weak suppressive effect of Co, Cu, or Ni coproporphyrin I on the basal growth of strain ASN212 might provide a clue to developing preventive and therapeutic strategies for infectious disease caused by drug-resistant strains of *Mycobacteria*, *Listeria*, *Staphylococcus* species,<sup>15</sup> and diseases relating to the abundance of *Firmicutes* in the human gut microbiome.<sup>6,16</sup>

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2. To a solution of 2.0 mg (2.7  $\mu$ mol) of coproporphyrin I dihydrochloride (Sigma-Aldrich, USA) in

2.0 mL of DMSO, 1.5 mg (2.5 eq) of Zn(OAc)<sub>2</sub> in 0.2 mL of H<sub>2</sub>O was added. The resultant solution was stirred overnight at room temperature, and the major product was separated by HPLC (Mightysil RP-18 GP Aqua, MeCN (0.1% AcOH)/H<sub>2</sub>O (0.1%AcOH), 53:47) to give **1** in 66.7% yield. Cu or Zn complexes **1**, **2**, **9**, and **13** could be synthesized at room temperature, while heating at 100 °C was necessary for the other complexes **7**, **8**, **10**, **11**, **12**, and **14** whose yields were 57.9 – 81.8%. Coproporphyrin III dihydrochloride was purchased from Frontier Scientific Inc., USA, and the yield of **2** was 68.3%.

3. The culture conditions are essentially the same as those described in Ref. 1 except for the scale (96-well plate) and shaking speed (1,000 r.p.m.). Growth of strain ASN212 was evaluated according to the assay protocol of CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay (Promega, USA).
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