1 **A limited concentration range of diaphorin, a polyketide**

2 **produced by a bacterial symbiont of the Asian citrus psyllid,**

3 **promotes the *in vitro* gene expression with bacterial ribosomes**

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5 **(Running title: Diaphorin promotes bacterial gene expression *in vitro*)**

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7 (Observations: 1,200 words with a maximum of 2 figures and 25 references)

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Abstract (<250 words)

20 Diaphorin is a polyketide produced by 〝*Candidatus* Profftella armatura〟

21 (*Gammaproteobacteria*: *Burkholderiales*), an obligate symbiont of a devastating

22 agricultural pest, the Asian citrus psyllid *Diaphorina citri* (Hemiptera: Psyllidae).

23 Physiological concentrations of diaphorin, which *D. citri* contains at levels as high as 2–

24 20 mM, are inhibitory to various eukaryotes and *Bacillus subtilis* (*Firmicutes*: *Bacilli*)

25 but promote the growth and metabolic activity of *Escherichia coli*

26 (*Gammaproteobacteria*: *Enterobacterales*). Our previous study demonstrated that

27 five-millimolar diaphorin, which exhibits significant inhibitory and promoting effects

28 on cultured *B. subtilis* and *E. coli*, respectively, inhibits *in vitro* gene expression

29 utilizing purified *B. subtilis* and *E. coli* ribosomes. This suggested that the adverse

30 effects of diaphorin on *B. subtilis* are partly due to its influence on gene expression.

31 However, the result appeared inconsistent with the positive effects on *E. coli*. Moreover,

32 the diaphorin concentration in bacterial cells, where genes are expressed *in vivo*, may be

33 lower than in culture media. Therefore, the present study analyzed the effects of 50 and

34 500 µM of diaphorin on bacterial gene expression using the same analytical method.

35 The result revealed that this concentration range of diaphorin, in contrast to

36 five-millimolar diaphorin, promotes the *in vitro* translation with the *B. subtilis* and *E.*

37 *coli* ribosomes, suggesting that the positive effects of diaphorin on *E. coli* are due to its

38 direct effects on translation. This study demonstrated for the first time that a

39 pederin-type compound promotes gene expression, establishing a basis for utilizing its

40 potential in pest management and industrial applications.

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Importance (<150 words)

44 This study revealed that a limited concentration range of diaphorin, a secondary

45 metabolite produced by a bacterial symbiont of an agricultural pest, promotes cell-free

46 gene expression utilizing substrates and proteins purified from bacteria. The unique

47 property of diaphorin, which is inhibitory to various eukaryotes and *Bacillus subtilis* but

48 promotes the growth and metabolic activity of *Escherichia coli*, may affect the

49 microbial flora of the pest insect, potentially influencing the transmission of devastating

50 plant pathogens. Moreover, the activity may be exploited to improve the efficacy of

51 industrial production by *E. coli,* which is often used to produce various important

52 materials, including pharmaceuticals, enzymes, amino acids, and biofuels. This study

53 elucidated a part of the mechanism by which the unique activity of diaphorin is

54 expressed, constructing a foundation for applying the unique property to pest

55 management and industrial use.

56   Microbes utilize secondary metabolites to mediate interactions with neighboring

57   organisms. Such molecules exhibit diverse biological activities, some of which facilitate

58   symbiotic relationships between the microbes and their animal hosts (1, 2).

59   Diaphorin is a polyketide produced by "*Candidatus* Profftella armatura"

60   (*Gammaproteobacteria*: *Burkholderiales*), an intracellular symbiont harbored alongside

61   the primary symbiont "*Candidatus* Carsonella ruddii" (*Gammaproteobacteria*:

62   *Oceanospirillales*) (3, 4) in the bacteriome organ (5–7) of the Asian citrus psyllid

63   *Diaphorina citri* (Hemiptera: Psyllidae) (8–11). *D. citri* is a serious agricultural pest that

64   transmits "*Candidatus* Liberibacter" spp. (*Alphaproteobacteria*: *Rhizobiales*), the

65   pathogens of the most destructive and incurable citrus disease, huanglongbing (12, 13).

66   Conserved presence of *Profftella* and its diaphorin-synthesizing gene clusters in

67   *Diaphorina* spp. underline the physiological and ecological significance of diaphorin for

68   the host psyllids (14, 15). Diaphorin, which *D. citri* contains at a concentration as high

69   as 2–20 mM in the body (16), exerts inhibitory effects on various eukaryotes (8, 17, 18)

70   and *Bacillus subtilis* (*Firmicutes*: *Bacilli*) (19) but promotes the growth and metabolic

71   activity of *Escherichia coli* (*Gammaproteobacteria*: *Enterobacterales*) (19), implying

72   that this secondary metabolite serves as a defensive agent of the holobiont

73   (host-symbiont assemblage) against eukaryotes and some bacterial lineages but is

74   beneficial for other bacteria (8, 17, 19). Besides "*Ca.* Liberibacter" spp. and the

75   bacteriome-associated mutualists, *D. citri* may harbor various secondary symbionts of a

76   facultative nature, including *Wolbachia* (*Alphaproteobacteria*: *Rickettsiales*) and

77   *Arsenophonus* (*Gammaproteobacteria*: *Enterobacterales*) (14). Recent studies are

78   revealing that interactions among these bacterial populations are important for psyllid

79   biology and host plant pathology (10, 14, 20–22). In this context, the unique property of

80   diaphorin may affect the microbiota of *D. citri*, potentially influencing the transmission

81   of "*Ca.* Liberibacter" spp. Moreover, this unique activity of diaphorin may be exploited

82   to improve the efficacy of industrial production by *E. coli,* which is frequently used to

83   produce various important materials, including pharmaceuticals, enzymes, amino acids,

84   and biofuels (19).

85   Diaphorin belongs to the family of pederin-type compounds (8, 19), which

86   exhibit toxicity and antitumor activity by suppressing eukaryotic protein synthesis

87   through binding to the E-site of the 60S subunit of eukaryotic ribosomes (23). However,

88   little is known about the effects of these compounds on bacterial gene expression (24).

89   To explore the possibility that diaphorin exerts its distinct activity on bacteria by

90   directly targeting bacterial gene expression, our previous study analyzed the effects of

91   diaphorin on the *in vitro* gene expression using ribosomes isolated from *B. subtilis* and

92   *E. coli*, quantifying production of the super folder green fluorescent protein (sfGFP)

93   (25). Five-millimolar diaphorin was used for the analysis because this concentration

94   exhibited significant inhibitory and promoting effects on *B. subtilis* and *E. coli*,

95   respectively, in culture experiments (19). The result showed that five-millimolar

96   diaphorin inhibits gene expression involving ribosomes from both *B. subtilis* and *E. coli*,

97   suggesting that the adverse effects of diaphorin on *B. subtilis* are attributed to, at least

98   partly, its inhibitory effects on gene expression (25). On the other hand, the result did

99   not explain the promoting effects of diaphorin on *E. coli*. Moreover, the concentration

100   of diaphorin in the intracellular environment, where the inherent gene expression

101   machinery works, may be lower than in the culture medium. Therefore, in the present

102   study, we analyzed the effect of 50 and 500 µM of diaphorin on bacterial gene

103   expression using the same assay system.

104   Cell-free translation of sfGFP with diaphorin at final concentrations of 50 and

105   500 µM demonstrated that this concentration range of diaphorin promotes the *in vitro*

106   gene expression involving ribosomes of both *E. coli* and *B. subtilis* (Fig. 1). Namely,

107   the relative activity of gene expression using the *E. coli* ribosome treated with 50 µM

108   diaphorin was 1.079 ± 0.012 (mean ± standard error, $n = 48$), which was moderately

109   (7.9%) but significantly ($p < 0.001$, Steel test) higher than that of the control ($1.000 \pm$

5

110   0.008, n = 96, Fig. 1A). Furthermore, the relative gene expression activity using the *E.*

111   *coli* ribosome treated with 500 µM diaphorin was 1.089 ± 0.017 (n = 48), which was

112   again moderately (8.9%) but significantly (p < 0.001, Steel test) higher than that of the

113   control (Fig. 1A). These results imply that the positive effects of diaphorin on the

114   growth and metabolic activity of *E. coli* (19) can be attributed to its direct effects on the

115   core gene expression machinery. When cultured in media containing five-millimolar

116   diaphorin (19), *E. coli* may be able to keep the intracellular diaphorin concentration

117   within this range, positively affecting their vital activities. Regarding *B. subtilis*,

118   although the relative gene expression activity using the *B. subtilis* ribosome along with

119   50 µM diaphorin (0.992 ± 0.023, n = 48) was not significantly different (p > 0.05, Steel

120   test, Fig. 1B) from the control (1.000 ± 0.011, n = 96), the gene expression using the *B.*

121   *subtilis* ribosome with 500 µM diaphorin (1.084 ± 0.034, n = 48) was moderately

122   (8.4%) but significantly (p < 0.001, Steel test) higher than the control (Fig. 1B). This

123   result appears inconsistent with previously observed adverse effects of the same

124   concentration of diaphorin on the cultured *B. subtilis* (19). However, transmission

125   electron microscopy showed that diaphorin also damages the *B. subtilis* cell envelope

126   (19), which may negate the positive effects of the appropriate concentration of

127   diaphorin on the gene expression machinery of *B. subtilis*.

128   This study elucidated a part of the mechanism by which the unique activity of

129   diaphorin is expressed, constructing a foundation for applying the unique property of

130   diaphorin to pest management and industrial use. Moreover, this study demonstrated for

131   the first time that a pederin-type compound promotes the gene expression of organisms.

132

133   **Materials and methods**

134   **Preparation of diaphorin**

135   Diaphorin was extracted and purified as described previously (8, 17, 19, 25). Adult *D.*

136   *citri* were ground in methanol, and the extracts were purified using an LC10

137   high-performance liquid chromatography system (Shimadzu) with an Inertsil ODS-3

138   C18 reverse-phase preparative column (GL Science).

139

Preparation of the *Bacillus subtilis* ribosome

141   The *B. subtilis* ribosomes were purified as described previously (25). *B. subtilis* cells

142   were passed through a French press cell (Ohtake) at approximately 110 MPa (16,000

143   psi), and ribosomes were captured using HiTrap Butyl FF columns (Cytiva). The eluent

144   was ultracentrifuged (100,000 ×g, 4°C, 16 h) using Optima L-100 XP Ultracentrifuge

145   (Beckman Coulter) to sediment ribosomes.

146

Quantification of cell-free synthesis of sfGFP

148   The *in vitro* gene expression activities involving ribosomes of *E. coli* and *B. subtilis*

149   were evaluated utilizing a PURE*frex 2.0* kit (GeneFrontier) as previously described (25).

150   Reaction solutions of translation were separated by SDS-polyacrylamide gel

151   electrophoresis. After renaturation, the fluorescence of sfGFP was elicited at 488 nm,

152   passed through a 520 nm band pass filter, and recorded using a Typhoon 9400 image

153   analyzer (GE Healthcare). The fluorescence intensity of sfGFP was quantified using the

154   ImageQuant TL software (version 8.1, GE Healthcare).

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Statistical analysis

157   All statistical analyses were conducted using R version 4.1.3. Multiple comparisons

158   were conducted using the Kruskal-Wallis test followed by the Steel test.

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Acknowledgments

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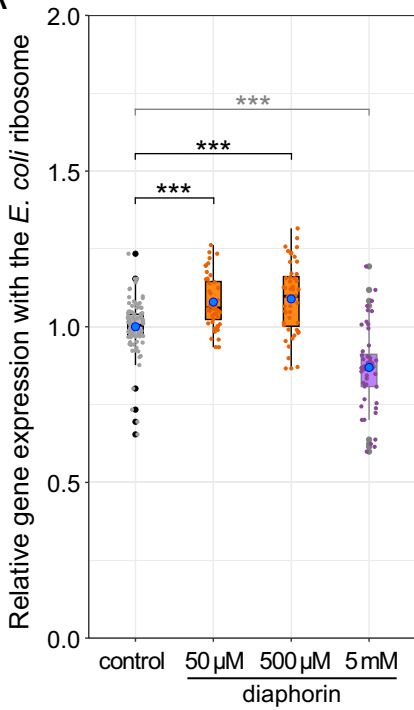
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Figure legends

Figure 1. Cell-free gene expression with bacterial ribosomes is promoted by a limited concentration range of diaphorin. (A) Relative gene expression with the *E. coli* ribosome. The signal intensity of synthesized sfGFP in each sample is normalized to the mean signal intensity of control samples. Jitter plots of all data points (control, *n* = 96; others, *n* = 48) and box plots (gray, control; orange, 50 μm and 500 μm diaphorin) showing their distributions (median, quartiles, minimum, and maximum) are indicated. Blue dots represent the mean. Asterisks indicate a statistically significant difference (***, *p* < 0.001, Steel test). For reference, previously published data of 5 mM-diaphorin treatment (19) are shown in purple dots (*n* = 48) with a box plot. (B) Relative gene expression with the *B. subtilis* ribosome. The signal intensity of synthesized sfGFP in each sample is normalized to the mean signal intensity of control samples. Jitter plots of all data points (control, *n* = 96; others, *n* = 48) and box plots (gray, control; green, 50 μm and 500 μm diaphorin) showing their distributions (median, quartiles, minimum, and maximum) are indicated. Blue dots represent the mean. Asterisks indicate a statistically significant difference (***, *p* < 0.001, Steel test). Previously published data of 5 mM-diaphorin treatment (19) are shown in purple dots (*n* = 48) and a box plot.

A

B