1   The dual role of TonB genes in turnerbactin uptake and carbohydrate

2   utilization in the shipworm symbiont *Teredinibacter turnerae*

3

4   Hiroaki Naka[1,2] and Margo G. Haygood[1]

5

6   [1]Department of Medicinal Chemistry, the University of Utah

7   [2]Division of Genetics, Oregon National Primate Research Center, Oregon Health & Science University

8

9

Abstract

11

12   *Teredinibacter turnerae* is an intracellular bacterial symbiont that resides in the gills of

13   shipworms, wood-eating bivalve mollusks. This bacterium produces a catechol

14   siderophore, turnerbactin, required for the survival of this bacterium under iron limiting

15   conditions. The turnerbactin biosynthetic genes are contained in one of the secondary

16   metabolite clusters conserved among *T. turnerae* strains. However, Fe(III)-turnerbactin

17   uptake mechanisms are largely unknown. Here, we show that the first gene of the

18   cluster, *fttA* a homologue of Fe(III)-siderophore TonB-dependent outer membrane

19   receptor (TBDR) genes is indispensable for iron uptake via the endogenous

20   siderophore, turnerbactin, as well as by an exogenous siderophore, amphi-enterobactin,

21   ubiquitously produced by marine vibrios. Furthermore, three TonB clusters containing

22   four *tonB* genes were identified, and two of these genes, *tonB1b* and *tonB2*, functioned

23   not only for iron transport but also for carbohydrate utilization when cellulose was a sole

24   carbon source. Gene expression analysis revealed that none of the *tonB* genes and

25   other genes in those clusters were clearly regulated by iron concentration while

26   turnerbactin biosynthesis and uptake genes were up-regulated under iron limiting

27   conditions, highlighting the importance of *tonB* genes even in iron rich conditions,

28   possibly for utilization of carbohydrates derived from cellulose.

29

Introduction

Iron is an essential nutrient for almost all living organisms including bacteria. However, available free iron is extremely limited in the marine environment due to its insolubility in the presence of oxygen, and in the host due to iron chelation by host iron-binding proteins; thus the amount of available free iron is much lower than the amount bacteria require for their proliferation. Therefore, bacteria have evolved active transport systems to sequester sufficient amounts of iron to survive and prosper in those environments (Crichton, 2016). One of these systems is siderophore-mediated iron transport. Siderophores are small molecule iron-chelating compounds synthesized by a nonribosomal peptide synthetase (NRPS) system or NRPS-independent pathway. Siderophores exported to external environments form stable complexes with ferric iron, and in Gram-negative bacteria, Fe^{3+}-siderophore complexes are transported to the bacterial cytosol via specific outer membrane receptors across the outer membrane, and ABC or MSF type siderophore transporters across inner membranes (Crosa and Walsh, 2002;Cuiv et al., 2004;Raymond and Dertz, 2004;Winkelmann, 2004;Hannauer et al., 2010;Reimmann, 2012). Gram-negative bacteria require TonB complexes typically composed of TonB, ExbB and ExbD, that locate in the inner membrane, to transduce energy derived from proton motive force to the Fe^{3+}-siderophore specific outer membrane receptors for their activity (Braun, 1995;Postle and Kadner, 2003;Noinaj et al., 2010). Although essential, an excess amount of iron is toxic due to its radical potential, therefore the expression of genes required for iron transport are tightly regulated by the concentration of iron to maintain a suitable cellular iron concentration (Andrews et al., 2003). It has also been demonstrated in many bacteria that iron influences not only the expression of iron metabolism genes but also acts as a signal that regulates the expression of genes that affect bacterial adaptation to environmental and/or host conditions (Crosa, 1997;Andrews et al., 2003;Fleischhacker and Kiley, 2011).

Shipworms of the family Teredinidae are marine bivalve mollusks most of which bore wood and consume wood as a nutrient source (Turner, 1966;Distel et al., 2011). To utilize wood as a nutrient, insoluble lignocellulose needs to be broken down into

61   soluble forms of carbohydrate. This enzymatic activity relies on symbiotic

62   gammaproteobacteria that reside in bacteriocytes in the gills (Waterbury et al.,

63   1983;Distel et al., 2002a;Luyten et al., 2006;Ekborg et al., 2007). *Teredinibacter*

64   *turnerae* is the first bacterial symbiont isolated from shipworms. This bacterium

65   produces cellulolytic enzymes and fixes atmospheric nitrogen that could contribute to

66   shipworm metabolism in woody environments where the amount of nitrogen is restricted

67   (Distel et al., 2002b;Lechene et al., 2007;Altamia et al., 2014;O'Connor et al., 2014). *T.*

68   *turnerae* T7901 carries many secondary metabolite gene clusters and production of

69   bioactive compounds has been reported (Elshahawi et al., 2013;Han et al., 2013;Miller

70   et al., 2021;Miller et al., 2022). One of the secondary metabolite gene clusters, Region

71   7, carries the genes that are responsible for the biosynthesis of siderophore turnerbactin

72   (Han et al., 2013). Sequencing and metagenomic analysis revealed that the Region 7

73   cluster and its relatives were found to fall within the gene cluster family GCF_8,

74   members of which occur in all *T. turnerae* strains sequenced as well as other shipworm

75   symbiotic bacteria, indicating the importance of this cluster for the physiology of

76   shipworm symbiotic bacteria (Altamia et al., 2020). The *tnbF* gene encoding a non-

77   ribosomal peptide synthetase in this cluster was shown to be essential for the

78   biosynthesis of turnerbactin and survival of this bacterium under iron limiting conditions

79   (Han et al., 2013). Turnerbactin was detected in the shipworm, *Lyrodus pedicellatus*,

80   harboring *T. turnerae*, suggesting the potential importance of turnerbactin in the

81   symbiotic state. *T. turnerae* might have elevated iron requirements due to the need to

82   synthesize iron rich nitrogenase (Han et al., 2013). It has been reported that *T. turnerae*

83   carries two TonB gene clusters, TonB2 and TonB3, that resemble clusters found in

84   marine vibrios although the function of those genes has yet to be characterized (Kuehl

85   and Crosa, 2010). In this work we show the essential role of the *fttA* gene encoding the

86   Fe^{3+}-turnerbactin outer membrane receptor for iron acquisition in *T. turnerae*.

87   Additionally, two of four *tonB* genes in the genome were indispensable for growth under

88   iron limiting conditions. These *tonB* genes were further found to be necessary for the

89   efficient growth of *T. turnerae* when cellulose was used as a sole carbon source.

90   Furthermore, we report that *tonB* genes in *T. turnerae* T7901 are not clearly regulated

91   by iron as compared with other iron transport-related genes, suggesting that *T. turnerae*

92 requires TonB genes even under iron rich condition to utilize carbohydrate(s) originating

93 from cellulose.

94

95

Materials and Methods

97

Strains, plasmids and growth media

99 Bacterial strains and plasmids used in this study are listed in **Table S1** while PCR

100 primers are listed in **Table S2**. *Teredinibacter turnerae* strains were cultured at 30 °C in

101 a modified chemically-defined shipworm basal medium (SBM) containing NaCl (17.94

102 gm/L), NH_4Cl (250 mg/L), Na_2SO_4 (3.01 gm/L), $NaHCO_3$ (0.147 gm/L), Na_2CO_3 (10.5

103 mg/L), KCl (0.5 gm/L), KBr (73.5 mg/L), H_3BO_3 (22.36 mg/L), $SrCl_2 \cdot 6H_2O$ (18 mg/L),

104 KH_2PO_4 (15.24 mg/L), $C_6H_8O_7$ (2.75 mg/L), NaF (2.25 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (2.4

105 mg/L), $MnCl_2 \cdot 4H_2O$ (1.81 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.22 mg/L), $CuSO_4 \cdot 5H_2O$ (0.079 mg/L),

106 $Co(NO_3)_2 \cdot 6H_2O$ (0.049 mg/L), HEPES (4.77 gm/L, pH = 8.0), and appropriate amounts

107 of carbon sources. $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$ and ferric ammonium citrate (FAC) were

108 supplemented in the medium. Sucrose (0.5 %), cellulose (sigmacell 101)(0.2 %), and

109 carboxymethylcellulose (0.5 %) were used as carbon sources, and agar (1 %) was

110 added to prepare solid media. Under our standard growth conditions, which include 50

111 μM of $MgCl_2 \cdot 6H_2O$ and 10 μM of $CaCl_2 \cdot 2H_2O$, cell aggregation was observed. However,

112 we found that by a reducing the concentration of $MgCl_2 \cdot 6H_2O$ (0.05 μM) and

113 $CaCl_2 \cdot 2H_2O$ (0.5 μM) in the SBM medium, *T. turnerae* grew without aggregation. *E. coli*

114 strains were cultured in LB broth or agar. Thymidine at 0.3 mM (f/c) was supplemented

115 for the growth of *E. coli* π3813. When required, antibiotics were supplemented in the

116 growth medium at the following concentration: ampicillin (Amp) at 100 μg/ml for *E. coli*,

117 kanamycin (Km) at 50 μg/ml for *E. coli* and *T. turnerae* and carbenicillin (Carb) at 100

118 μg/ml for *T. turnerae*.

119

Construction of plasmids

121 The plasmid pHN31(pDM4-Km), used for mutant construction was constructed as

122 follows. The kanamycin resistance cassette from pBBR1MCS-2 (Kovach et al., 1995)

123   was PCR-amplified using Km-F-*EcoR*V and Km-R-*EcoR*V primers, and ligated into T-

124   vectors. After confirming the nucleotide sequences, the Km cassette was cloned into the

125   *EcoR*V site of pDM4 (Milton et al., 1996), generating pHN31.

126   To express genes in *T. turnerae,* we used the pHN33(pPROBE-tacP-GenP)

127   plasmid constructed as follows. pMMB208 was digested with *Sca*I and *Age*I, and the

128   DNA fragment containing *lac*I, the *tac* promoter and a multiple cloning site was ligated

129   into the corresponding restriction enzyme sites of pPROBE'-gfp[ASV] (Miller et al.,

130   2000), generating pHN32. The DNA fragment containing the gentamicin resistance

131   gene promoter from pBBR1MCS-3 (Kovach et al., 1995) were PCR-amplified using

132   primers, GenP-F-*Hind*III and GenP-R-*Sal*I, and cloned into T-vector. After confirmation

133   of the nucleotide sequence, the plasmid was digested by *Hind*III and *Sal*I, and the

134   promoter sequence was ligated in the corresponding restriction enzyme sites of pHN32

135   plasmid, generating pHN33**.** pPROBE-gfp[ASV] was a gift from Steven Lindow

136   (Addgene plasmid # 40166 ; http://n2t.net/addgene:40166 ; RRID:Addgene_40166)

137

138   **Mutant construction and complementation**

139   DNA fragments of upstream and downstream regions of the target genes to be mutated

140   were combined by splicing by overhang extension PCR with modification as described

141   before (Senanayake and Brian, 1995;Naka et al., 2018), and the PCR-amplified

142   fragments were ligated into pGEM-T easy (Promega). After sequence confirmation, the

143   deletion fragments were ligated into the corresponding restriction enzyme sites of

144   pHN31. The plasmids thus obtained were transformed into *E. coli* strains S17-1λpir or

145   π3813 (thymidine auxotroph), and conjugated into *T. turnerae* T7901. When *E. coli*

146   π3813 was used, thymidine (f/c 0.3 mM) was supplemented to the growth medium, and

147   *E. coli* π3813 that carries pEVS104 (Stabb and Ruby, 2002) was used as a conjugation

148   helper strain. To counterselect *E. coli*, 1st recombinants were selected by plating

149   exconjugants on SBM-N-cellulose plates (for S17-1λpir conjugation) with Km (50 µg/ml)

150   or SBM-N-sucrose without thymidine plates (for π3813 conjugation) supplemented with

151   Km (50 µg/ml). 1st recombinants thus obtained were grown in liquid medium without

152   antibiotics, streaked on SBM-N containing 15% sucrose, and incubated until colonies

153   were formed. The deletion mutants were obtained by screening the colonies that were

154   sensitive to Km, by colony PCR using primers. To complement mutants, DNA

155   fragments that contain wild type genes and their potential ribosomal binding sites were

156   PCR-amplified, and cloned into T-vectors. After sequence confirmation, the DNA

157   fragments were cloned into pHN33, and the plasmid was conjugated into *T. turnerae* as

158   described above.

159

160   **RNA extraction**

161   All glassware was soaked in a 10% hydrochloric acid bath then rinsed with milliQ water,

162   to remove iron. *T. turnerae* T7901 and its derivatives were grown in iron limiting (L-

163   SBM-N-sucrose with 0.1 μM FAC) and iron rich (L-SBM-N-sucrose with 10 μM FAC)

164   until exponential phase (OD_{600} 0.2-0.3), and cell pellets were resuspended in TRIzol

165   Reagent (Invitrogen), and the samples were kept in a -80 °C freezer until processed.

166   Total RNAs were extracted by the Trizol-RNeasy hybrid protocol (Lopez and Bohuski,

167   2007). During RNA extraction, contaminated DNA was digested by 3 times treatment

168   with RNase-Free DNase Set (Qiagen), and the absence of DNA contamination in

169   extracted RNA was confirmed by PCR.

170

171   **Quantitative RT-PCR**

172   cDNA was synthesized from total RNA (1 μg) as a template using Superscript III

173   Reverse Transcriptase and random hexamer primers (Invitrogen), and quantitative PCR

174   were performed by StepOnePlus™ Fast Real-Time PCR System (Applied Biosystems)

175   using Power SYBR™ Green PCR Master Mix (Applied Biosystems). The fold change of

176   gene expression in two different conditions was measured by calculating ΔΔCt values

177   as described in (Livak and Schmittgen, 2001).

178

179

180   **Results**

181

182   **Characterization of the Fe-turnerbactin outer membrane receptor gene, *fttA***

183   One of the secondary metabolite clusters, Region 7 of *T. turnerae* T7901 contains

184   nonribosomal peptide synthetase (NRPS) genes (**Figure 1)**, and the major NRPS gene,

185   *tnbF* was shown to be essential for the siderophore turnerbactin production (Han et al.,

186   2013). The first gene of Region 7, *TERTU_RS18025* (old locus tag, *TERTU_4055*) was

187   annotated as a homologue of the TonB dependent outer membrane receptor (TBDR)g

188   gene, CCD03052, from *Azospirillum brasilense* Sp245 (Han et al., 2013). Further

189   comparison of the predicted amino acid sequence of *TERTU_RS18025* (named *fttA* in

190   this study) with known Fe^{3+}-siderophore outer membrane receptors revealed that FttA

191   shows similarity to *E. coli fepA* (27% identity/44% similarity in amino acid level) and

192   *Vibrio anguillarum fetA* (30% identity/48% similarity in amino acid level), suggesting its

193   potential role as a Fe^{3+}-turnerbactin uptake receptor. Although the TBDRss play an

194   essential role for the iron uptake in bacteria, there are cases in which Fe^{3+}-siderophores

195   can be transported via multiple TBDRs encoded by genes that reside in different

196   chromosomal loci (Poole et al., 1993;Rabsch et al., 1999;Mey et al., 2002;Ghysels et

197   al., 2004;Hannauer et al., 2010;Naka and Crosa, 2012;Wyckoff et al., 2015;Payne et al.,

198   2016). To investigate the role of the *fttA* gene, we constructed an in-frame *fttA* deletion

199   mutant, and the growth of the *fttA* mutant was compared with the wild type strain and

200   turnerbactin biosynthetic mutant (Δ*tnbF*), under iron rich and limiting conditions. As

201   shown in **Figure 2,** the Δ*fttA* mutant did not grow under the iron limiting condition as

202   compared with the wild type strain while this mutant still grew well in the iron rich growth

203   condition. The growth of the Δ*fttA* mutant under the iron limiting condition was

204   recovered when the *fttA* gene was expressed *in trans* in the *fttA* mutant confirming that

205   the growth defect was due to the deletion of the *fttA* gene (**Figure 3**). These results

206   indicate that the *fttA* gene is essential for the growth of *T. turnerae* under iron limiting

207   conditions.

208   To further investigate whether the growth deficiency of the Δ*fttA* mutant is due to

209   the failure of Fe^{3+}-turnerbactin uptake, we performed a bioassay (siderophore cross-

210   feeding assay). We first constructed a turnerbactin production deficient strain,

211   Δ*tnbA*Δ*tnbF*. The *tnbA* gene was also mutated to eliminate the 2,3-dihydroxybenzoate-

212   2,3-dehydrogenase (2,3-DHBA) production since 2,3-DHBA also acts as an iron

213   chelator (Bellaire et al., 2003). The *fttA* gene was mutated in the Δ*tnbA*Δ*tnbF*

214   background. Supplementation of the iron chelator, ethylenediamine-di-(o-hydroxyphenyl

215   acetic acid) (EDDA) into growth medium led to the failure of the growth of the

216   turnerbactin production deficient strains, Δ*tnbA*Δ*tnbF* and Δ*tnbA*Δ*tnbF*Δ*fttA* (**Figure 4**).

217   This growth defect of Δ*tnbA*Δ*tnbF* was overcome when the wild type strain producing

218   turnerbactin was spotted on the agar plate containing Δ*tnbA*Δ*tnbF* (see the growth halo

219   around the spot). However, the Δ*tnbA*Δ*tnbF*Δ*fttA* strain in which the *fttA* gene was

220   deleted didn't recover its growth in the presence of the wild type strain spot while

221   spotting ferric ammonium citrate was able to recover its growth. These results indicate

222   that the *fttA* gene is essential for the uptake of turnerbactin produced by the wild type

223   strain. Furthermore, to test whether *T. turnerae* T7901 can utilize an exogenous

224   siderophore produced by marine bacterium *Vibrio campbellii*, we used extracts obtained

225   from wild type *V. campbellii* that produces amphi-enterobactin and anguibactin, and its

226   derivatives, an amphi-enterobactin producer and an anguibactin producer (Naka et al.,

227   2018). Extracts rather than cultures were used because *V. campbellii* strains cannot

228   grow on SBM medium. The growth of *T. turnerae* was recovered when amphi-

229   enterobactin was provided by the indicator strain while anguibactin was not able to

230   compensate for the growth defect under iron limiting conditions. These results indicate

231   that *T. turnerae* can take up amphi-enterobactin but not anguibactin produced by the

232   marine pathogenic bacterium *V. campbellii*.

233

234   **Identification of TonB clusters in *T. turnerae* T7901**

235   The presence of the TonB2 and TonB3 clusters in *T. turnerae* was briefly described

236   before and those are similar to the TonB2 and TonB3 clusters of marine vibrios such as

237   *V. vulnificus* (Kuehl and Crosa, 2010)**,** but the function of those *tonB* genes has not

238   been elucidated yet. By sequence similarity searching of protein sequences annotated

239   in *T. turnerae* T7901 with well-characterized TonB genes from *E. coli* K-12, and marine

240   bacteria including *V. vulnificus*, *V. cholerae*, *V. anguillarum* and *Aeromonas hydrophila*,

241   we identified two more *tonB* gene homologues in a cluster (named here TonB1 cluster)

242   in addition to TonB2 and TonB3 clusters. Interestingly, the TonB1 cluster carries two

243   TonB genes, *tonB1a* and *tonB1b*, located next to each other and an *exbD* gene

244   homologue (*exbD1*), but an *exbB* homologue was not found in this cluster (**Figure 1**).

245   The TonB1 clusters in vibrios (consisting of *tonB1*, *exbB1* and *exbD1)* are located linked

246   to the heme/hemoglobin transport cluster (Occhino et al., 1998;O'Malley et al.,

247   1999;Stork et al., 2004;Wang et al., 2008;Kustusch et al., 2012). However, there is no

248   heme cluster near the TonB1 system in *T. turnerae*. Prediction of transmembrane

249   helices with TMHMM server version 2

250   (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) (Krogh et al., 2001)

251   indicated that TonB1b, TonB2 and TonB3 harbor one transmembrane domain typically

252   found in classical TonB proteins while TonB1a is an unusual TonB protein that carries

253   an extended N-terminal domain predicted to carry four transmembrane domains, that

254   can be found small number of bacteria (Chu et al., 2007).

255

256   **Two TonB genes are essential for the growth of *T. turnerae* under iron limiting**

257   **growth conditions**

258   To understand which TonB gene(s) facilitate the growth of *T. turnerae* under specified

259   conditions, single and multiple *tonB* gene mutants were constructed. Since *tonB1a* and

260   *tonB1b* genes are co-located, both *tonB1a* and *tonB1b* were deleted together,

261   generating the Δ*tonB1ab* mutant. The growth of those strains was compared under iron

262   rich and limiting growth conditions. As shown in **Figure 2**, single mutants that lack *tonB*

263   gene(s) in each TonB cluster such as Δ*tonB1ab*, Δ*tonB2* and Δ*tonB3* as well as the

264   double *tonB* gene mutants in the TonB1 and TonB3 cluster (Δ*tonB1ab*Δ*tonB3*) and in

265   the TonB2 and TonB3 cluster (*tonB2*Δ*tonB3*) showed growth under both iron rich and

266   limiting growth conditions. On the other hand, the *tonB* gene mutants in both the TonB1

267   and TonB2 cluster, Δ*tonB1ab*Δ*tonB2* and the quadruple *tonB* gene mutant in which all

268   *tonB* genes were eliminated, Δ*tonB1ab*Δ*tonB2*Δ*tonB3*, did not grow under iron limiting

269   conditions. Similar results were observed in the turnerbactin biosynthetic deficient

270   mutant Δ*tnbF* and the ferric-turnerbactin transport deficient Δ*fttA* mutant. These results

271   indicate that the *tonB* genes in both the TonB1 and TonB2 cluster are involved in the

272   iron transport in *T. turnerae* T7901.

273   We further performed complementation experiments to confirm that the growth

274   defect of some of mutants were not due to polar effects and/or secondary mutations,

275   and also to understand which *tonB1* genes (*tonB1a* or *tonB1b*) is responsible for the

276   growth of *T. turnerae* T7901 under iron limiting conditions. *tonB* genes with their

277   ribosomal binding sites were cloned in the expression vector pHN33, and conjugated

278   into the Δ*tonB1ab*Δ*tonB2*Δ*tonB3* mutant. The expression of all four TonB genes was

279   confirmed by RT-PCR (**Figure S1**). As shown in **Figure 3**, the growth of the quadruple

280   *tonB* mutant under iron limiting growth conditions were recovered only when *tonB1b* or

281   *tonB2* genes are expressed *in trans* in the quadruple *tonB* mutant. All strains grew well

282   under an iron rich growth condition. From these results, we conclude that out of four

283   *tonB* genes, *tonB1b* and *tonB2* are responsible for the growth of *T. turnerae* T7901

284   under iron limiting conditions, and *tonB1a* and *tonB3* are not responsible for iron uptake

285   under this growth condition.

286

287   **Involvement of TonB genes in the growth of *T. turnerae* T7901 cellulose as a**

288   **carbon source**

289   During the course of mutant construction in TonB genes, it was very hard to obtain the

290   Δ*tonB1ab*Δ*tonB2* mutant. We realized that this Δ*tonB1ab*Δ*tonB2* mutant does not grow

291   when cellulose is used as a sole carbon source in the growth medium. This mutant did

292   not show a growth defect on sucrose plates. Since supplementation of cellulose and

293   carboxymethyl cellulose (cellulose derivative) into the growth medium resulted in same

294   consequences, we decided to use carboxymethyl cellulose due to its solubility in growth

295   medium. We further tested the growth of all single and multiple *tonB* gene mutants on

296   SBM medium supplemented with either sucrose or carboxymethyl cellulose as a carbon

297   source, and we found that the mutants missing *tonB* genes in the both TonB1 and

298   TonB2 clusters (Δ*tonB1ab*Δ*tonB2*) and the strain that lacks all *tonB* genes

299   (Δ*tonB1ab*Δ*tonB2*Δ*tonB3*) showed a dramatic growth defect when carboxymethyl

300   cellulose was used as a sole carbon source (**Figure 2**). The rest of the mutants tested

301   grew on both sucrose and cellulose media. The growth defect in the quadruple TonB

302   mutant, Δ*tonB1ab*Δ*tonB2*Δ*tonB3* was recovered when the *tonB1b* or *tonB2* genes were

303   expressed *in trans* in the mutant while *tonB1a* and *tonB3* was not able to compensate

304   the growth defect on cellulose plates (**Figure 3**). These results demonstrate that *tonB1b*

305   and *tonB2* are involved in carbohydrate utilization when cellulose is provided as a sole

306   carbon source. Turnerbactin biosynthesis (Δ*tnbF*) and transport (Δ*fttA*) deficient mutants

307   did not show growth defects on cellulose plates, therefore the growth defect appears to

308   be independent of turnerbactin production and utilization.

309

Iron regulation of turnerbactin biosynthesis and transport genes

It has been proposed that Region 7 consists of two iron-regulated transcriptional units and both operons might be regulated by the ferric uptake regulator since two possible Fur binding sites (Fur boxes) were identified in the upstream regions of *fttA* and *tnbC* (Han et al., 2013). We performed RT-qPCR analysis to test whether genes in Region 7 are actually iron-regulated. Our results clearly showed that three representative genes such as *tnbA*, *tnbF* and *fttA* are up-regulated under iron limiting growth conditions (**Figure 5**). We also performed a Fur titration assay to test whether the *E. coli* ferric uptake regulator (Fur) binds to these putative TonB boxes. The result in **Figure S2** shows that *E. coli* Fur can bind to two Fur boxes as compared with two negative controls, indicating that Fur is involved in the up-regulation of those genes under iron limiting conditions.

Iron regulation of TonB genes

In many bacteria, TonB genes are typically regulated by iron to control internal iron concentration (Young and Postle, 1994;Zhao and Poole, 2000;Bosch et al., 2002;Ochsner et al., 2002;Bjarnason et al., 2003;Beddek et al., 2004;Osorio et al., 2004;Stork et al., 2004;Mey et al., 2005;Bender et al., 2007;Wang et al., 2008). To test iron-regulation of TonB genes in *T. turnerae*, we performed RT-qPCR using primers to amplify each TonB gene (**Figure 5**). The results indicate that relative expression levels of all TonB genes were not changed as much as those of *tnbA* and *tnbF* which were dramatically increased under iron limiting conditions as compared with iron rich conditions.

Discussion

One of the compounds *Teredinibacter turnerae* produces is the siderophore turnerbactin that is used to acquire iron which is an essential metal for their growth in iron limiting environments. It has been suggested that turnerbactin might be used to compete for

340   iron with casually associated environmental bacteria to survive under iron limiting

341   conditions which are typically found in marine environments and inside hosts (Han et

342   al., 2013). Turnerbactin-related genes were found in the secondary metabolite cluster,

343   Region 7 located within GCF_8 (identified by metagenomics), and the *tnbF* gene is

344   essential for turnerbactin biosynthesis (Han et al., 2013;Altamia et al., 2020). However,

345   the transport mechanism of Fe(III)-turnerbactin was not characterized yet. To transport

346   Fe(III)-siderophore across the outer membrane, Gram negative bacteria require the

347   TonB system that typically consists of TonB, ExbB and ExbD, that transduce proton

348   motive force generated in the inner membrane to outer membrane receptors, resulting

349   in conformational change in the outer membrane receptors (Ratliff et al., 2022). The

350   TonB system was originally found and has been extensively characterized in *E. coli*

351   (Postle and Larsen, 2007). *E. coli* and many other bacteria carry a single set of the

352   TonB system, but after finding two TonB systems in *V. cholerae* (Occhino et al., 1998),

353   multiple TonB systems have been identified and characterized in a number of bacteria,

354   including many *Vibrio* species (2 or 3 systems) (Seliger et al., 2001;Stork et al., 2004;Alice

355   et al., 2008;Wang et al., 2008;Tanabe et al., 2012), *Aeromonas hydrophila* (3 systems)

356   (Dong et al., 2016;Dong et al., 2019;Dong et al., 2023), *Pseudomonas aeruginosa* (3

357   systems) (Poole et al., 1996;Zhao and Poole, 2000;Huang et al., 2004), *Acinetobacter*

358   *baumannii* (3 systems) (Zimbler et al., 2013;Runci et al., 2019), and *Bacteroides fragilis*

359   (6 systems) (Parker et al., 2022). In those examples, some TonB systems are

360   functionally independent while others show functionally redundancy, for transport for

361   particular substances such as siderophores and other nutrients, or physiological

362   activities.

363   The aim of this study is to explain the Fe(III)-turnerbactin uptake mechanism. The

364   *fttA* gene located in Region 7 is a homologue of Fe(III)-siderophore TonB-dependent

365   outer membrane receptors (TBDRs). RT-qPCR analysis showed that the *fttA* gene as

366   well as two turnerbactin biosynthetic genes, *tnbA* and *tnbF* are clearly up-regulated

367   under iron limiting growth conditions. Iron-regulation of genes in Region 7 were further

368   analyzed by RNA sequencing (RNA-seq), and all annotated iron transport-related genes

369   in Region 7, *fttA* to *TERTU_RS18085* are up-regulated under iron limiting conditions

370   (**Table S3**). Furthermore, the Fur titration assay (FURTA) showed that the *E. coli* ferric

371   uptake regulator, Fur, can bind to the potential promoter regions previously identified

372   and located upstream of *fttA* and *tnbC* whereas the upstream region of

373   *TERTU_RS18075* showed a negative result. Taken together, the iron regulation of

374   Region 7 is caused by at least two distinct promoters in a Fur-dependent manner, as

375   proposed before (Han et al., 2013). We constructed an in-frame deletion mutant of *fttA*,

376   and showed that the *fttA* gene is responsible for Fe(III)-turnerbactin transport and

377   indispensable for growth under iron-limiting conditions while the *fttA* mutant grew well

378   under iron-rich growth conditions, demonstrating that FttA is the sole TBDR involved in

379   Fe(III)-turnerbactin uptake. We also tested the ability of *T. turnerae* to transport

380   xenosiderophores, amphi-enterobactin and anguibactin, from *Vibrio campbellii*. Our

381   results showed that *T. turnerae* can utilize Fe(III)-amphi-enterobactin or its hydrolyzed

382   derivatives as an iron source and it was independent of *fttA*, whereas Fe(III)-anguibactin

383   failed to enhance the growth of *T. turnerae* under iron limiting conditions. Amphi-

384   enterobactin is produced by both *V. campbellii* and *V. harveyi* that are members of the

385   Harveyi clade ubiquitously found in marine environments and some strains are

386   causative agents of vibriosis that affect marine vertebrates and invertebrates. On the

387   other hand, anguibactin is produced by *V. campbellii* but not by *V. harveyi* (Zane et al.,

388   2014;Naka et al., 2018). Our findings indicate that *T. turnerae* possess the ability to

389   "steal" iron from the siderophore or its derivatives commonly found in different species

390   rather than from the species specific siderophore, and this might provide an advantage

391   to *T. turnerae* to survive in marine environments where amphi-enterobactin is available.

392   It is still unknown what gene(s) is encoding the outer membrane receptor for Fe(III)-

393   amphi-enterobactin since *fttA* was not required for Fe(III)-amphi-enterobactin utilization.

394   In the *T. turnerae* T7901 genome, 38 genes were annotated to encode TonB-dependent

395   outer membrane receptors, and our RNA-seq result indicated that 6 genes in addition to

396   *fttA* were up-regulated (logFC > 1) under iron-limiting growth conditions (**Table S4**), and

397   one or some of them might be responsible for Fe(III)-amphi-enterobactin utilization.

398   By searching in the genome of *T. turnerae*, we identified four TonB genes that

399   are located in three TonB clusters. The *tonB1* cluster of *T. turnerae* is a unique *tonB*

400   cluster that contains two *tonB* genes, *tonB1a* and *tonB1b,* and *exbD1*, but lacks *exbB*

401   typically found in TonB clusters. TonB1a carries a N-terminal extension as compared

402 with conventional TonB proteins, and this type of TonB protein was identified by

403 bioinformatic analysis but the function is still unknown (Chu et al., 2007). The gene

404 organization of TonB2 and TonB3 clusters resemble marine vibrios and contain

405 homologues of *ttpB*, *ttpC*, *exbB*, *exbD*, *tonB* and *ttpD* in which *ttpB*, *ttpC* and *ttpD* are

406 specifically found in vibrios and some marine bacteria (Kuehl and Crosa, 2010;Barnes

407 et al., 2020). In vibrios, the TonB2 cluster is involved in iron transport while the function

408 of the TonB3 cluster is still unknown (Alice and Crosa, 2012;Duong-Nu et al., 2016).

409 The similarity of TonB2 and TonB3 systems, especially the presence of *ttpB*, *ttpC* and

410 *ttpD*, to those of vibrios indicate that the *tonB2* system could provide benefits to adapt in

411 coastal waters where both *T. turnerae* and vibrios live. Conversely, the TonB1 cluster of

412 *T. turnerae* did not show similarity to that of vibrios. *Vibrio* TonB1 systems are linked to

413 gene clusters that are responsible for hemin/hemoglobin uptake, and are involved in

414 hemin/hemoglobin uptake (Occhino et al., 1998;O'Malley et al., 1999;Mourino et al.,

415 2004;Lemos and Osorio, 2007;Wang et al., 2008;Septer et al., 2011;Kustusch et al.,

416 2012). We speculate that *T. tunerae* did not evolve a similar TonB1 cluster possibly due

417 to the absence of a heme/hemoglobin cluster, and *T. turnerae* does not encounter

418 environments in which hemin and/or hemoglobin is available during their life cycle, due

419 to the absence of hemoglobin in bivalves such as the shipworm hosts.

420   In most bacteria, TonB genes are normally up-regulated in iron limiting

421 conditions (actually repressed under iron rich conditions) because excess amounts of

422 iron are toxic to bacteria because it leads to Fenton reaction causing the overproduction

423 of reactive oxygen species in presence of oxygen. Interestingly, RT-qPCR results

424 showed that none of TonB genes as well as other genes in *tonB* clusters are clearly

425 regulated under iron limiting conditions and the expression pattern of TonB genes were

426 further confirmed by RNA-seq, supporting the result of RT-qPCR and also suggesting

427 that the regulation occurs at a cluster level. It has been reported that *tonB3* genes in *V.*

428 *vulnificus* and *A. hydrophila* are not iron regulated (Alice and Crosa, 2012;Dong et al.,

429 2019). However, neither of the *tonB3* genes in those bacteria are involved in iron

430 transport. It is of interest that all *T. turnerae* TonB genes are not clearly iron-regulated

431 even though *tonB1b* and *tonB2* genes *are* involved in Fe(III)-turnerbactin utilization.

432   These results indicated that *T. turnerae* might still need *tonB* genes expressed even in

433   iron rich conditions.

434   One of the unusual features of *T. turnerae* is its ability to degrade lignocellulose

435   from wood and utilize its derivatives as a carbon source (Waterbury et al., 1983;Distel et

436   al., 2002b). It has been reported that some bacteria use TBDRs to take up plant-derived

437   carbohydrates, mono- and poly-saccharides. *Xanthomonas campestris* pv. *campestris*

438   (Xcc) use TBDR to take up sucrose, and the comparative genomic and gene expression

439   analysis suggested that Xcc as well as some marine bacteria possibly take up plant

440   carbohydrates via TBDRs (Blanvillain et al., 2007). *Caulobacter crescentus* uses the

441   TonB1 system to transport maltose and maltodextrins (Neugebauer et al.,

442   2005;Lohmiller et al., 2008). We showed that two of the *tonB* genes, *tonB1b* and *tonB2*,

443   are involved in carbohydrate utilization derived from cellulose in *T. turnerae* whereas

444   mutations in other *tonB* genes (*tonB1a* and *tonB3*) did not affect the growth. These

445   results indicate that the same set of *tonB1b* and *tonB2* are functional not only for Fe(III)-

446   turnerbactin uptake but also cellulose utilization. Further studies are required to identify

447   TBDRs involved in the uptake of cellulose-derived carbohydrates, and what

448   carbohydrate(s) are transported across the outer membrane. Some TBDRs are located

449   close to genes potentially involved in hemicellulose degradation (Data not shown). It is

450   worth noting that the experiments were performed under iron rich conditions, therefore

451   the lack of growth was not due to iron limitation. These results indicate that *tonB1b* and

452   *tonB2* are functional even under iron rich conditions. All *tonB* genes are expressed in

453   both iron rich and limiting growth conditions (**Table S3**). This dual role of TonB1b and

454   TonB2 for iron and carbohydrate uptake could explain why *T. turnerae* does not clearly

455   regulate those genes depending on iron concentrations although TonB genes are down-

456   regulated under iron rich conditions in most bacteria.

457   In summary, the *fttA* gene, a homologue of Fe(III)-siderophore TBDR genes, is

458   indispensable for the survival of *T. turnerae* under iron limiting growth conditions

459   because it is essential for Fe(III)-turnerbactin utilization as an iron source. FttA appears

460   to be essential for the transport of Fe(III)-turnerbactin across the outer membrane, and

461   Fe(III)-amphi-enterobactin produced by other marine bacteria can be utilized as an iron

462   source without FttA. Two out of four *tonB* genes, *tonB1b* and *tonB2*, show functional

463   redundancy for the survival of *T. turnerae* under iron limiting conditions as well as the

464   growth of *T. turnerae* when cellulose was supplied as a sole carbon source. Since *tonB*

465   genes are known to energize TBDRs to substrate import across the outer membrane,

466   those findings indicate that carbohydrates derived from cellulose are likely transported

467   by TBDRs. All of genes in Region 7 encompassing *fttA* to *TERTU_RS18085* were

468   repressed under iron rich conditions to avoid intracellular excess iron whereas the

469   expression of the *tonB* genes is remained under iron rich conditions, indicating the

470   importance of *tonB* genes even under iron rich conditions possibly for the utilization of

471   cellulose as a carbon source.

472

473

474   **Figure legends**

475

476   **Figure 1**. Gene clusters involved in siderophore-mediated iron transport.

477   Panel A. the turnerbactin biosynthesis and transport cluster. Panel B. Three *tonB*

478   clusters. The figure was modified from a gene cluster map constructed by Gene

479   Graphics (Harrison et al., 2018). Green arrows indicate genes already characterized or

480   predicted to be responsible for siderophore biosynthesis. Orange arrows indicate genes

481   annotated to be involved in Fe(III)-siderophore uptake, and of those, TonB-homologues

482   are shown in red color.

483

484   **Figure 2**. Involvement of *fttA* and TonB genes in iron transport and carbohydrate

485   derived from cellulose.

486   Panel A, Growth of *T. turnerae* mutants under different growth conditions.

487   Sucrose (0.5 %) or carboxymethyl cellulose (0.5 %) were added as a sole carbon

488   source in the SBM agar plates, and FAC (1μM) and EDDA (10 μM), and FAC (10 μM)

489   were supplemented in the SBM medium to obtain iron limiting and rich conditions,

490   respectively. *T. turnerae* strains were streaked on the plates, and the pictures were

491   taken after 7 days incubation at 30 °C. FAC, ferric ammonium citrate; EDDA,

492   ethylenediamine-N,N′-bis(2-hydroxyphenylacetic acid)

493   Panel B, Growth response of *tonB* deletions to iron restriction and carboxymethyl

494   cellulose combined.

495   "wt" indicates the presence of wild type *tonB* genes while "Δ" shows the absence of the

496   tonB gene (the in-frame gene deletion). The strains that grew under iron limiting

497   conditions when sucrose was used as a carbon source or when carboxymethyl cellulose

498   was used as a carbon source under iron rich conditions were highlighted as green and

499   shown as "+" while the strains that did not grow were highlighted as red and shown as "-

500   ".

501

502   **Figure 3**. Complementation of *fttA* and *tonB* mutants

503   Sucrose or carboxymethyl cellulose were added as a sole carbon source in the SBM

504   agar plates. FAC (1μM) and EDDA (10 μM), and FAC (10 μM) were added in the SBM

505   medium to obtain iron limiting and rich conditions, respectively. *T. turnerae* strains were

506   streaked on the plates, and the pictures were taken after 7 days incubation at 30 °C.

507   FAC, ferric ammonium citrate; EDDA, ethylenediamine-N,N′-bis(2-hydroxyphenylacetic

508   acid. pHN45, plasmid expression vector;

509   tonBs- , Δ*tonB1ab*Δ*tonB2*Δ*tonB3*

510

511   **Figure 4**. Bioassay to test the involvement of FttA in transport of endogenous and

512   exogenous siderophores. Δ*tnbA*Δ*tnbF* produces neither turnerbactin nor its precursor

513   due to the mutation in *tnbF* and *tnbA*, respectively, therefore cannot grow under iron

514   limiting growth condition generated by supplementing an iron chelator, ethylenediamine-

515   N,N′-bis(2-hydroxyphenylacetic acid. *T. turnerae* strains were grown under non-

516   aggregation conditions. T7901, 5 μl of *T. turnerae* T7901 culture (containing

517   turnerbactin); Amphi-ent, extracts containing amphi-enterobactin obtained from *V.*

518   *campbellii* HY01Δ*angR* (Naka et al., 2018); extracts contain anguibactin prepared from

519   *V. campbellii* HY01Δ*aebF* (Naka et al., 2018); Fe, 5μl of ferric ammonium citrate.

520   Pictures were taken after 7 days incubation at 30°C.

521

522   **Figure 5**. Regulation of iron transport-related genes in *T. turnerae* T7901

523 Expression of genes between iron limiting and rich growth conditions were compared by

524 qRT-PCR. The data represents the mean value of at least three biological replicates

525 with error bars that are the standard error of the mean.

526

527

Acknowledgements

529

534

535

References

537

538 Alice, A.F., and Crosa, J.H. (2012). The TonB3 system in the human pathogen Vibrio vulnificus

539   is under the control of the global regulators Lrp and cyclic AMP receptor protein. *J*

540   *Bacteriol* 194, 1897-1911.

541 Alice, A.F., Naka, H., and Crosa, J.H. (2008). Global gene expression as a function of the iron

542   status of the bacterial cell: influence of differentially expressed genes in the virulence of

543   the human pathogen Vibrio vulnificus. *Infect Immun* 76, 4019-4037.

544 Altamia, M.A., Lin, Z., Trindade-Silva, A.E., Uy, I.D., Shipway, J.R., Wilke, D.V., Concepcion,

545   G.P., Distel, D.L., Schmidt, E.W., and Haygood, M.G. (2020). Secondary Metabolism in

546   the Gill Microbiota of Shipworms (Teredinidae) as Revealed by Comparison of

547   Metagenomes and Nearly Complete Symbiont Genomes. *mSystems* 5.

548 Altamia, M.A., Wood, N., Fung, J.M., Dedrick, S., Linton, E.W., Concepcion, G.P., Haygood,

549   M.G., and Distel, D.L. (2014). Genetic differentiation among isolates of Teredinibacter

550   turnerae, a widely occurring intracellular endosymbiont of shipworms. *Mol Ecol* 23, 1418-

551   1432.

552 Andrews, S.C., Robinson, A.K., and Rodriguez-Quinones, F. (2003). Bacterial iron homeostasis.

553   *FEMS Microbiol Rev* 27, 215-237.

554 Barnes, A.D., Pfeifer, H.J., Zbylicki, B.R., Roberts, E.K., Rudd, J.C., Manzo, M.A., Phillips, E.A.,
555   Berry, M.M., and Kenton, R.J. (2020). Two novel proteins, TtpB2 and TtpD2, are
556   essential for iron transport in the TonB2 system of Vibrio vulnificus. *Microbiologyopen* 9,
557   e00947.

558 Beddek, A.J., Sheehan, B.J., Bosse, J.T., Rycroft, A.N., Kroll, J.S., and Langford, P.R. (2004).
559   Two TonB systems in Actinobacillus pleuropneumoniae: their roles in iron acquisition
560   and virulence. *Infect Immun* 72, 701-708.

561 Bellaire, B.H., Elzer, P.H., Baldwin, C.L., and Roop, R.M., 2nd (2003). Production of the
562   siderophore 2,3-dihydroxybenzoic acid is required for wild-type growth of Brucella
563   abortus in the presence of erythritol under low-iron conditions in vitro. *Infect Immun* 71,
564   2927-2832.

565 Bender, K.S., Yen, H.C., Hemme, C.L., Yang, Z., He, Z., He, Q., Zhou, J., Huang, K.H., Alm,
566   E.J., Hazen, T.C., Arkin, A.P., and Wall, J.D. (2007). Analysis of a ferric uptake regulator
567   (Fur) mutant of Desulfovibrio vulgaris Hildenborough. *Appl Environ Microbiol* 73, 5389-
568   5400.

569 Bjarnason, J., Southward, C.M., and Surette, M.G. (2003). Genomic profiling of iron-responsive
570   genes in Salmonella enterica serovar typhimurium by high-throughput screening of a
571   random promoter library. *J Bacteriol* 185, 4973-4982.

572 Blanvillain, S., Meyer, D., Boulanger, A., Lautier, M., Guynet, C., Denance, N., Vasse, J.,
573   Lauber, E., and Arlat, M. (2007). Plant carbohydrate scavenging through tonB-
574   dependent receptors: a feature shared by phytopathogenic and aquatic bacteria. *PLoS
575   One* 2, e224.

576 Bosch, M., Garrido, E., Llagostera, M., Perez De Rozas, A.M., Badiola, I., and Barbe, J. (2002).
577   Pasteurella multocida exbB, exbD and tonB genes are physically linked but
578   independently transcribed. *FEMS Microbiol Lett* 210, 201-208.

579 Braun, V. (1995). Energy-coupled transport and signal transduction through the gram-negative
580   outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. *FEMS Microbiol
581   Rev* 16, 295-307.

582 Chu, B.C., Peacock, R.S., and Vogel, H.J. (2007). Bioinformatic analysis of the TonB protein
583   family. *Biometals* 20, 467-483.

584 Crichton, R.R. (2016). *Iron metabolism : from molecular mechanisms to clinical consequences.*
585   John Wiley & Sons.

586 Crosa, J.H. (1997). Signal transduction and transcriptional and posttranscriptional control of
587   iron-regulated genes in bacteria. *Microbiol Mol Biol Rev* 61, 319-336.

588   Crosa, J.H., and Walsh, C.T. (2002). Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. *Microbiol Mol Biol Rev* 66**,** 223-249.

590   Cuiv, P.O., Clarke, P., Lynch, D., and O'connell, M. (2004). Identification of rhtX and fptX, novel genes encoding proteins that show homology and function in the utilization of the siderophores rhizobactin 1021 by Sinorhizobium meliloti and pyochelin by Pseudomonas aeruginosa, respectively. *J Bacteriol* 186**,** 2996-3005.

594   Distel, D.L., Amin, M., Burgoyne, A., Linton, E., Mamangkey, G., Morrill, W., Nove, J., Wood, N., and Yang, J. (2011). Molecular phylogeny of Pholadoidea Lamarck, 1809 supports a single origin for xylotrophy (wood feeding) and xylotrophic bacterial endosymbiosis in Bivalvia. *Mol Phylogenet Evol* 61**,** 245-254.

598   Distel, D.L., Beaudoin, D.J., and Morrill, W. (2002a). Coexistence of multiple proteobacterial endosymbionts in the gills of the wood-boring Bivalve Lyrodus pedicellatus (Bivalvia: Teredinidae). *Appl Environ Microbiol* 68**,** 6292-6299.

601   Distel, D.L., Morrill, W., Maclaren-Toussaint, N., Franks, D., and Waterbury, J. (2002b). Teredinibacter turnerae gen. nov., sp. nov., a dinitrogen-fixing, cellulolytic, endosymbiotic gamma-proteobacterium isolated from the gills of wood-boring molluscs (Bivalvia: Teredinidae). *Int J Syst Evol Microbiol* 52**,** 2261-2269.

605   Dong, Y., Geng, J., Liu, J., Pang, M., Awan, F., Lu, C., and Liu, Y. (2019). Roles of three TonB systems in the iron utilization and virulence of the Aeromonas hydrophila Chinese epidemic strain NJ-35. *Appl Microbiol Biotechnol* 103**,** 4203-4215.

608   Dong, Y., Liu, J., Pang, M., Du, H., Wang, N., Awan, F., Lu, C., and Liu, Y. (2016). Catecholamine-Stimulated Growth of Aeromonas hydrophila Requires the TonB2 Energy Transduction System but Is Independent of the Amonabactin Siderophore. *Front Cell Infect Microbiol* 6**,** 183.

612   Dong, Y., Xu, M., Wan, X., Zhao, D., Geng, J., Huang, H., Jiang, M., Lu, C., and Liu, Y. (2023). TonB systems are required for Aeromonas hydrophila motility by controlling the secretion of flagellin. *Microbes Infect* 25**,** 105038.

615   Duong-Nu, T.M., Jeong, K., Hong, S.H., Nguyen, H.V., Ngo, V.H., Min, J.J., Lee, S.E., and Rhee, J.H. (2016). All Three TonB Systems Are Required for Vibrio vulnificus CMCP6 Tissue Invasiveness by Controlling Flagellum Expression. *Infect Immun* 84**,** 254-265.

618   Ekborg, N.A., Morrill, W., Burgoyne, A.M., Li, L., and Distel, D.L. (2007). CelAB, a multifunctional cellulase encoded by Teredinibacter turnerae T7902T, a culturable symbiont isolated from the wood-boring marine bivalve Lyrodus pedicellatus. *Appl Environ Microbiol* 73**,** 7785-7788.

622   Elshahawi, S.I., Trindade-Silva, A.E., Hanora, A., Han, A.W., Flores, M.S., Vizzoni, V., Schrago,
623   C.G., Soares, C.A., Concepcion, G.P., Distel, D.L., Schmidt, E.W., and Haygood, M.G.
624   (2013). Boronated tartrolon antibiotic produced by symbiotic cellulose-degrading bacteria
625   in shipworm gills. *Proc Natl Acad Sci U S A* 110, E295-304.

626   Fleischhacker, A.S., and Kiley, P.J. (2011). Iron-containing transcription factors and their roles
627   as sensors. *Curr Opin Chem Biol* 15, 335-341.

628   Ghysels, B., Dieu, B.T.M., Beatson, S.A., Pirnay, J.P., Ochsner, U.A., Vasil, M.L., and Cornelis,
629   P. (2004). FpvB, an alternative type I ferripyoverdine receptor of Pseudomonas
630   aeruginosa. *Microbiology (Reading)* 150, 1671-1680.

631   Han, A.W., Sandy, M., Fishman, B., Trindade-Silva, A.E., Soares, C.A., Distel, D.L., Butler, A.,
632   and Haygood, M.G. (2013). Turnerbactin, a novel triscatecholate siderophore from the
633   shipworm endosymbiont Teredinibacter turnerae T7901. *PLoS One* 8, e76151.

634   Hannauer, M., Barda, Y., Mislin, G.L., Shanzer, A., and Schalk, I.J. (2010). The ferrichrome
635   uptake pathway in Pseudomonas aeruginosa involves an iron release mechanism with
636   acylation of the siderophore and recycling of the modified desferrichrome. *J Bacteriol*
637   192, 1212-1220.

638   Harrison, K.J., Crecy-Lagard, V., and Zallot, R. (2018). Gene Graphics: a genomic
639   neighborhood data visualization web application. *Bioinformatics* 34, 1406-1408.

640   Huang, B., Ru, K., Yuan, Z., Whitchurch, C.B., and Mattick, J.S. (2004). tonB3 is required for
641   normal twitching motility and extracellular assembly of type IV pili. *J Bacteriol* 186, 4387-
642   4389.

643   Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., 2nd, and
644   Peterson, K.M. (1995). Four new derivatives of the broad-host-range cloning vector
645   pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166, 175-176.

646   Krogh, A., Larsson, B., Von Heijne, G., and Sonnhammer, E.L. (2001). Predicting
647   transmembrane protein topology with a hidden Markov model: application to complete
648   genomes. *J Mol Biol* 305, 567-580.

649   Kuehl, C.J., and Crosa, J.H. (2010). The TonB energy transduction systems in Vibrio species.
650   *Future Microbiol* 5, 1403-1412.

651   Kustusch, R.J., Kuehl, C.J., and Crosa, J.H. (2012). The ttpC gene is contained in two of three
652   TonB systems in the human pathogen Vibrio vulnificus, but only one is active in iron
653   transport and virulence. *J Bacteriol* 194, 3250-3259.

654   Lechene, C.P., Luyten, Y., Mcmahon, G., and Distel, D.L. (2007). Quantitative imaging of
655   nitrogen fixation by individual bacteria within animal cells. *Science* 317, 1563-1566.

656   Lemos, M.L., and Osorio, C.R. (2007). Heme, an iron supply for vibrios pathogenic for fish.
657   *Biometals* 20**,** 615-626.

658   Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-
659   time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25**,** 402-408.

660   Lohmiller, S., Hantke, K., Patzer, S.I., and Braun, V. (2008). TonB-dependent maltose transport
661   by Caulobacter crescentus. *Microbiology (Reading)* 154**,** 1748-1754.

662   Lopez, J.A., and Bohuski, E. (2007). Total RNA extraction with TRIZOL reagent and purification
663   with QIAGEN RNeasy Mini Kit.© DGC. *Bloomington, IN: Indiana University*.

664   Luyten, Y.A., Thompson, J.R., Morrill, W., Polz, M.F., and Distel, D.L. (2006). Extensive
665   variation in intracellular symbiont community composition among members of a single
666   population of the wood-boring bivalve Lyrodus pedicellatus (Bivalvia: Teredinidae). *Appl*
667   *Environ Microbiol* 72**,** 412-417.

668   Mey, A.R., Wyckoff, E.E., Kanukurthy, V., Fisher, C.R., and Payne, S.M. (2005). Iron and fur
669   regulation in Vibrio cholerae and the role of fur in virulence. *Infect Immun* 73**,** 8167-8178.

670   Mey, A.R., Wyckoff, E.E., Oglesby, A.G., Rab, E., Taylor, R.K., and Payne, S.M. (2002).
671   Identification of the Vibrio cholerae enterobactin receptors VctA and IrgA: IrgA is not
672   required for virulence. *Infect Immun* 70**,** 3419-3426.

673   Miller, B.W., Lim, A.L., Lin, Z., Bailey, J., Aoyagi, K.L., Fisher, M.A., Barrows, L.R., Manoil, C.,
674   Schmidt, E.W., and Haygood, M.G. (2021). Shipworm symbiosis ecology-guided
675   discovery of an antibiotic that kills colistin-resistant Acinetobacter. *Cell Chem Biol* 28**,**
676   1628-1637 e1624.

677   Miller, B.W., Schmidt, E.W., Concepcion, G.P., and Haygood, M.G. (2022). Halogenated Metal-
678   Binding Compounds from Shipworm Symbionts. *J Nat Prod* 85**,** 479-484.

679   Miller, W.G., Leveau, J.H., and Lindow, S.E. (2000). Improved gfp and inaZ broad-host-range
680   promoter-probe vectors. *Mol Plant Microbe Interact* 13**,** 1243-1250.

681   Milton, D.L., O'toole, R., Horstedt, P., and Wolf-Watz, H. (1996). Flagellin A is essential for the
682   virulence of Vibrio anguillarum. *J Bacteriol* 178**,** 1310-1319.

683   Mourino, S., Osorio, C.R., and Lemos, M.L. (2004). Characterization of heme uptake cluster
684   genes in the fish pathogen Vibrio anguillarum. *J Bacteriol* 186**,** 6159-6167.

685   Naka, H., and Crosa, J.H. (2012). Identification and characterization of a novel outer membrane
686   protein receptor FetA for ferric enterobactin transport in Vibrio anguillarum 775 (pJM1).
687   *Biometals* 25**,** 125-133.

688   Naka, H., Reitz, Z.L., Jelowicki, A.M., Butler, A., and Haygood, M.G. (2018). Amphi-enterobactin
689   commonly produced among Vibrio campbellii and Vibrio harveyi strains can be taken up

690   by a novel outer membrane protein FapA that also can transport canonical Fe(III)-

691   enterobactin. *J Biol Inorg Chem* 23, 1009-1022.

692 Neugebauer, H., Herrmann, C., Kammer, W., Schwarz, G., Nordheim, A., and Braun, V. (2005).

693   ExbBD-dependent transport of maltodextrins through the novel MalA protein across the

694   outer membrane of Caulobacter crescentus. *J Bacteriol* 187, 8300-8311.

695 Noinaj, N., Guillier, M., Barnard, T.J., and Buchanan, S.K. (2010). TonB-dependent

696   transporters: regulation, structure, and function. *Annu Rev Microbiol* 64, 43-60.

697 O'connor, R.M., Fung, J.M., Sharp, K.H., Benner, J.S., Mcclung, C., Cushing, S., Lamkin, E.R.,

698   Fomenkov, A.I., Henrissat, B., Londer, Y.Y., Scholz, M.B., Posfai, J., Malfatti, S., Tringe,

699   S.G., Woyke, T., Malmstrom, R.R., Coleman-Derr, D., Altamia, M.A., Dedrick, S.,

700   Kaluziak, S.T., Haygood, M.G., and Distel, D.L. (2014). Gill bacteria enable a novel

701   digestive strategy in a wood-feeding mollusk. *Proc Natl Acad Sci U S A* 111, E5096-

702   5104.

703 O'malley, S.M., Mouton, S.L., Occhino, D.A., Deanda, M.T., Rashidi, J.R., Fuson, K.L., Rashidi,

704   C.E., Mora, M.Y., Payne, S.M., and Henderson, D.P. (1999). Comparison of the heme

705   iron utilization systems of pathogenic Vibrios. *J Bacteriol* 181, 3594-3598.

706 Occhino, D.A., Wyckoff, E.E., Henderson, D.P., Wrona, T.J., and Payne, S.M. (1998). Vibrio

707   cholerae iron transport: haem transport genes are linked to one of two sets of tonB,

708   exbB, exbD genes. *Mol Microbiol* 29, 1493-1507.

709 Ochsner, U.A., Wilderman, P.J., Vasil, A.I., and Vasil, M.L. (2002). GeneChip expression

710   analysis of the iron starvation response in Pseudomonas aeruginosa: identification of

711   novel pyoverdine biosynthesis genes. *Mol Microbiol* 45, 1277-1287.

712 Osorio, C.R., Lemos, M.L., and Braun, V. (2004). Identification of Fur regulated genes in the

713   bacterial fish pathogen Photobacterium damselae ssp. piscicida using the Fur titration

714   assay. *Biometals* 17, 725-733.

715 Parker, A.C., Seals, N.L., Baccanale, C.L., and Rocha, E.R. (2022). Analysis of Six tonB Gene

716   Homologs in Bacteroides fragilis Revealed That tonB3 is Essential for Survival in

717   Experimental Intestinal Colonization and Intra-Abdominal Infection. *Infect Immun* 90,

718   e0046921.

719 Payne, S.M., Mey, A.R., and Wyckoff, E.E. (2016). Vibrio Iron Transport: Evolutionary

720   Adaptation to Life in Multiple Environments. *Microbiol Mol Biol Rev* 80, 69-90.

721 Poole, K., Neshat, S., Krebes, K., and Heinrichs, D.E. (1993). Cloning and nucleotide sequence

722   analysis of the ferripyoverdine receptor gene fpvA of Pseudomonas aeruginosa. *J*

723   *Bacteriol* 175, 4597-4604.

724   Poole, K., Zhao, Q., Neshat, S., Heinrichs, D.E., and Dean, C.R. (1996). The Pseudomonas
725   aeruginosa tonB gene encodes a novel TonB protein. *Microbiology (Reading)* 142 (Pt
726   6)**,** 1449-1458.

727   Postle, K., and Kadner, R.J. (2003). Touch and go: tying TonB to transport. *Mol Microbiol* 49**,**
728   869-882.

729   Postle, K., and Larsen, R.A. (2007). TonB-dependent energy transduction between outer and
730   cytoplasmic membranes. *Biometals* 20**,** 453-465.

731   Rabsch, W., Voigt, W., Reissbrodt, R., Tsolis, R.M., and Baumler, A.J. (1999). Salmonella
732   typhimurium IroN and FepA proteins mediate uptake of enterobactin but differ in their
733   specificity for other siderophores. *J Bacteriol* 181**,** 3610-3612.

734   Ratliff, A.C., Buchanan, S.K., and Celia, H. (2022). The Ton Motor. *Front Microbiol* 13**,** 852955.

735   Raymond, K.N., and Dertz, E.A. (2004). "Biochemical and Physical Properties of Siderophores,"
736   in *Iron Transport in Bacteria,* eds. J.H. Crosa, A.R. Mey & S.M. Payne. John Wiley &
737   Sons, Inc), 1-17.

738   Reimmann, C. (2012). Inner-membrane transporters for the siderophores pyochelin in
739   Pseudomonas aeruginosa and enantio-pyochelin in Pseudomonas fluorescens display
740   different enantioselectivities. *Microbiology (Reading)* 158**,** 1317-1324.

741   Runci, F., Gentile, V., Frangipani, E., Rampioni, G., Leoni, L., Lucidi, M., Visaggio, D., Harris,
742   G., Chen, W., Stahl, J., Averhoff, B., and Visca, P. (2019). Contribution of Active Iron
743   Uptake to Acinetobacter baumannii Pathogenicity. *Infect Immun* 87.

744   Seliger, S.S., Mey, A.R., Valle, A.M., and Payne, S.M. (2001). The two TonB systems of Vibrio
745   cholerae: redundant and specific functions. *Mol Microbiol* 39**,** 801-812.

746   Senanayake, S.D., and Brian, D.A. (1995). Precise large deletions by the PCR-based overlap
747   extension method. *Mol Biotechnol* 4**,** 13-15.

748   Septer, A.N., Wang, Y., Ruby, E.G., Stabb, E.V., and Dunn, A.K. (2011). The haem-uptake
749   gene cluster in Vibrio fischeri is regulated by Fur and contributes to symbiotic
750   colonization. *Environ Microbiol* 13**,** 2855-2864.

751   Stabb, E.V., and Ruby, E.G. (2002). RP4-based plasmids for conjugation between Escherichia
752   coli and members of the Vibrionaceae. *Methods Enzymol* 358**,** 413-426.

753   Stork, M., Di Lorenzo, M., Mourino, S., Osorio, C.R., Lemos, M.L., and Crosa, J.H. (2004). Two
754   tonB systems function in iron transport in Vibrio anguillarum, but only one is essential for
755   virulence. *Infect Immun* 72**,** 7326-7329.

756   Tanabe, T., Funahashi, T., Shiuchi, K., Okajima, N., Nakao, H., Miyamoto, K., Tsujibo, H., and
757   Yamamoto, S. (2012). Characterization of Vibrio parahaemolyticus genes encoding the

758   systems for utilization of enterobactin as a xenosiderophore. *Microbiology (Reading)*
759   158, 2039-2049.

760 Turner, R.D. (1966). *A survey and illustrated catalogue of the Teredinidae (Mollusca: Bivalvia).*
761   Cambridge,: Museum of Comparative Zoology, Harvard University.

762 Wang, Q., Liu, Q., Cao, X., Yang, M., and Zhang, Y. (2008). Characterization of two TonB
763   systems in marine fish pathogen Vibrio alginolyticus: their roles in iron utilization and
764   virulence. *Arch Microbiol* 190, 595-603.

765 Waterbury, J.B., Calloway, C.B., and Turner, R.D. (1983). A cellulolytic nitrogen-fixing bacterium
766   cultured from the gland of deshayes in shipworms (bivalvia: teredinidae). *Science* 221,
767   1401-1403.

768 Winkelmann, G. (2004). "Ecology of Siderophores," in *Iron Transport in Bacteria,* eds. J.H.
769   Crosa, A.R. Mey & S.M. Payne. John Wiley & Sons, Inc), 435-450.

770 Wyckoff, E.E., Allred, B.E., Raymond, K.N., and Payne, S.M. (2015). Catechol Siderophore
771   Transport by Vibrio cholerae. *J Bacteriol* 197, 2840-2849.

772 Young, G.M., and Postle, K. (1994). Repression of tonB transcription during anaerobic growth
773   requires Fur binding at the promoter and a second factor binding upstream. *Mol*
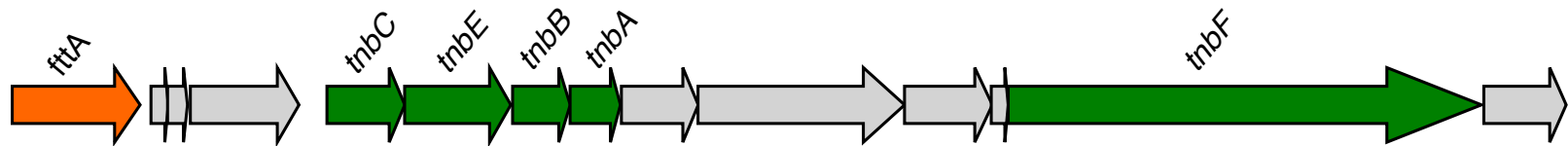774   *Microbiol* 11, 943-954.

775 Zane, H.K., Naka, H., Rosconi, F., Sandy, M., Haygood, M.G., and Butler, A. (2014).
776   Biosynthesis of amphi-enterobactin siderophores by Vibrio harveyi BAA-1116:
777   identification of a bifunctional nonribosomal peptide synthetase condensation domain. *J*
778   *Am Chem Soc* 136, 5615-5618.

779 Zhao, Q., and Poole, K. (2000). A second tonB gene in Pseudomonas aeruginosa is linked to
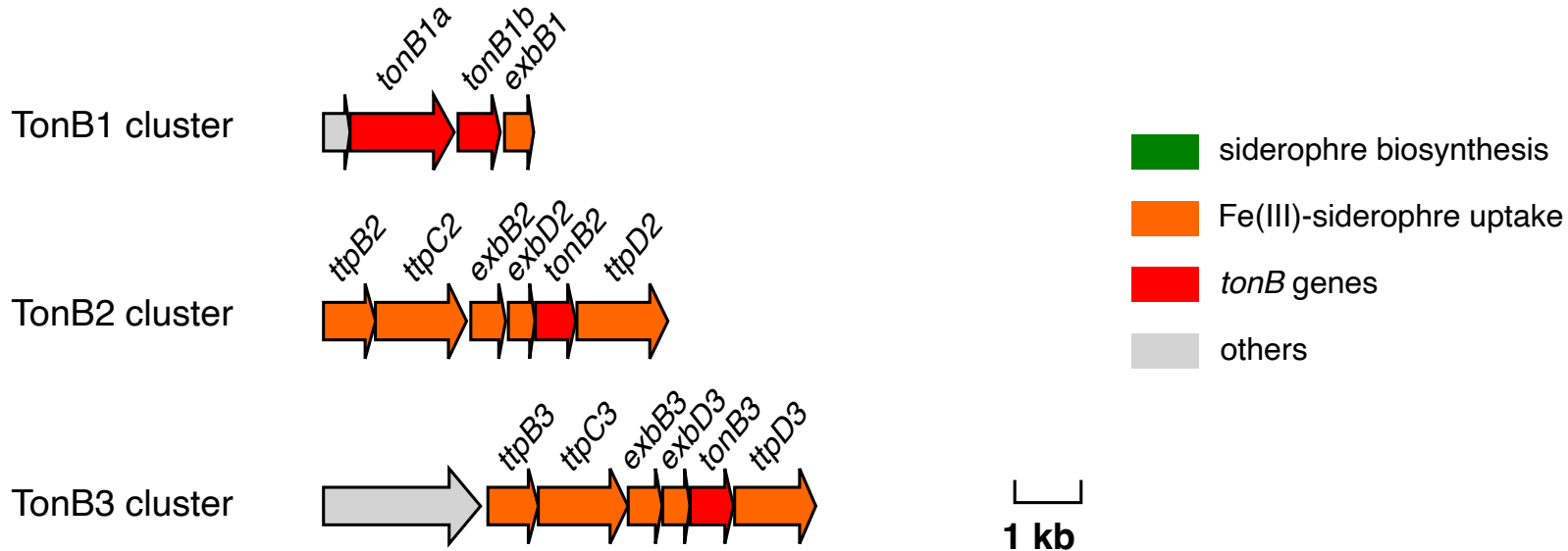780   the exbB and exbD genes. *FEMS Microbiol Lett* 184, 127-132.

781 Zimbler, D.L., Arivett, B.A., Beckett, A.C., Menke, S.M., and Actis, L.A. (2013). Functional
782   features of TonB energy transduction systems of Acinetobacter baumannii. *Infect Immun*
783   81, 3382-3394.

784

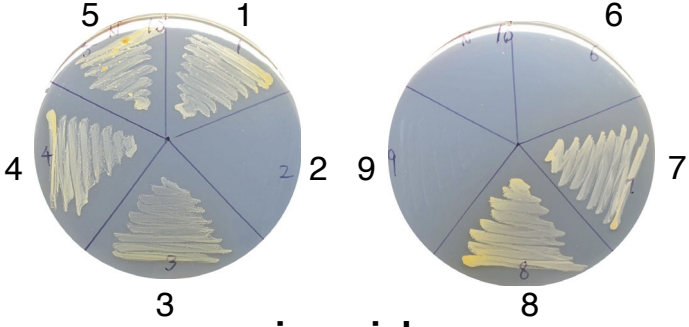**Figure 1**
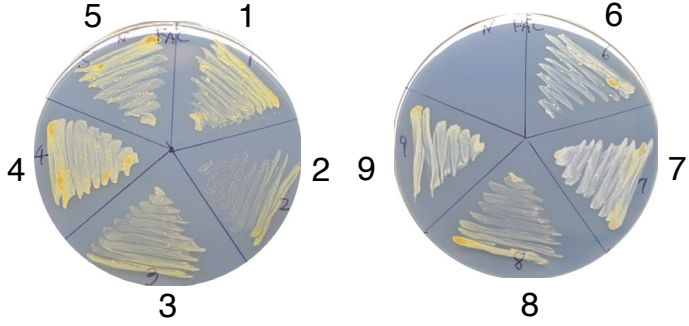
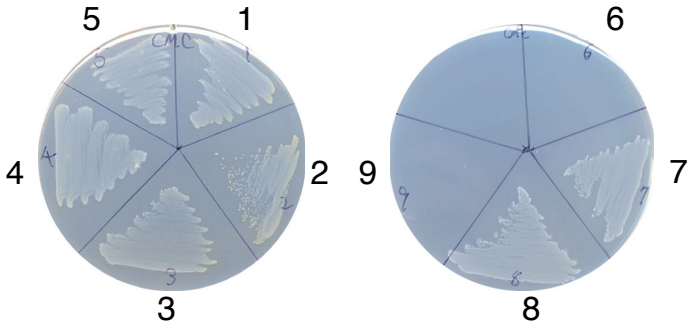**A. turnerbactin biosynthesis and transport cluster**

fttA *tnbC* *tnbE* *tnbB* *tnbA* *tnbF*

B. three TonB clusters

TonB1 cluster
tonB1a *tonB1b* *exbB1*

TonB2 cluster
ttpB2 *ttpC2* *exbB2* *exbD2* *tonB2* *ttpD2*

TonB3 cluster
ttpB3 *ttpC3* *exbB3* *exbD3* *tonB3* *ttpD3*

siderophre biosynthesis
Fe(III)-siderophre uptake
tonB genes
others

1 kb

Figure 2

A

iron limiting (sucrose)

iron rich (sucrose)

iron rich (carboxymethyl cellulose)

1. Wild type
2. Δ*fttA*
3. Δ*tonB1ab*
4. Δ*tonB2*
5. Δ*tonB3*
6. Δ*tonB1ab*Δ*tonB2*
7. Δ*tonB1ab*Δ*tonB3*
8. Δ*tonB2*Δ*tonB3*
9. Δ*tonB1ab*Δ*tonB2*Δ*tonB3*

B

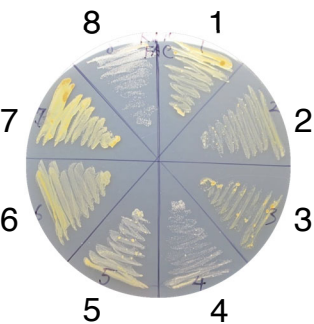|								
tonB gene	**1A**	Δ	wt	wt	Δ	wt	Δ	Δ
	1B	Δ	wt	wt	Δ	wt	Δ	Δ
	2	wt	Δ	wt	wt	Δ	Δ	Δ
	3	wt	wt	Δ	Δ	Δ	wt	Δ
Growth		+	+	+	+	+	-	-

Figure 3

Iron limiting (sucrose)

Iron rich (carboxymethyl cellulose)

Iron rich (sucrose)

1. Wild type (pHN45)
2. Δ*fttA* (pHN45)
3. Δ*fttA* (pHN45-*fttA*)
4. *tonBs⁻* (pHN45)
5. *tonBs⁻* (pHN45-*tonB1a*)
6. *tonBs⁻* (pHN45-*tonB1b*)
7. *tonBs⁻* (pHN45-*tonB2*)
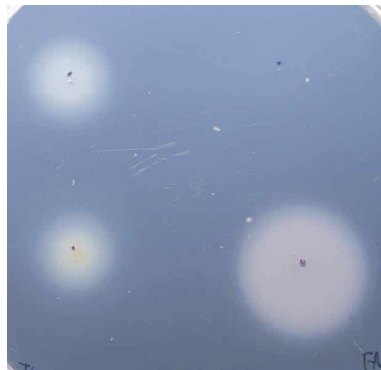8. *tonBs⁻* (pHN45-*tonB3*)

Figure 4

Indicator strains

ΔtnbAΔtnbF

ΔtnbAΔtnbFΔfttA

Amphi-ent   Ang

turnerbactin   Fe

Amphi-ent   Ang

turnerbactin   Fe

Figure 5

Fe limiting / Fe rich
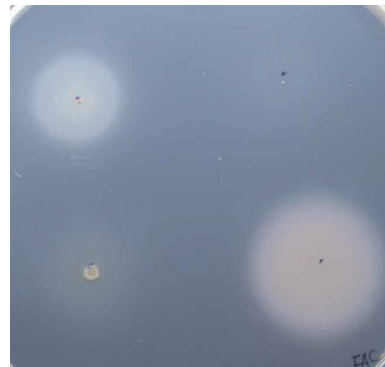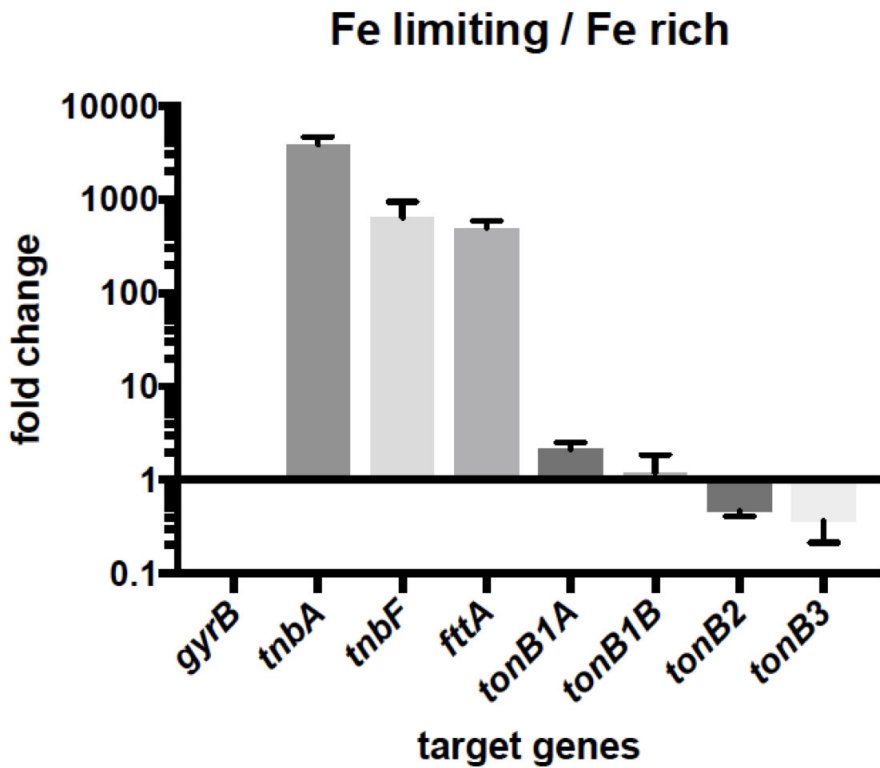
Table S1. Strains and plasmids used in this study

Strains and plasmids	Characteristics	Reference or source
***T. turnerae* strains**		
T7901 (ATCC 36867)	Wild-type strain isolated from *Bankia gouldi*	(Distel et al., 2002)
HNTT-1	T7901Δ*fttA*	This study
HNTT-2	T7901Δ*tnbF*	This study
HNTT-3	T7901Δ*tnbA*Δ*tnbF*	This study
HNTT-4	T7901Δ*tnbA*Δ*tnbF*Δ*fttA*	This study
HNTT-5	T7901Δ*tonB1ab*	This study
HNTT-6	T7901Δ*tonB2*	This study
HNTT-7	T7901Δ*tonB3*	This study
HNTT-8	T7901Δ*tonB1ab*Δ*tonB2*	This study
HNTT-9	T7901Δ*tonB1ab*Δ*tonB3*	This study
HNTT-10	T7901Δ*tonB2*Δ*tonB3*	This study
HNTT-11	T7901Δ*tonB1ab*Δ*tonB2*Δ*tonB3*	This study
HNTT-12	T7901Δ*tonB1ab*Δ*tonB2*Δ*tonB3*	This study
***Escherichia coli* strains**		
DH5α	F-, ϕ80*lacZ*ΔM15 *endA1 recA1 hsdR*17(r$_\kappa$−,m$_\kappa$+) *phoA supE*44 *thi*-1 *gyrA*96 *relA*1 Δ(*lacZYA-argF*)*U169* λ−	Laboratory stock

S17-1λ*pir*	λ-*pir* lysogen; *thi pro hsdR hsdM₊recA RP4 2-Tc*::*Mu-Km*::*Tn7*(Tpʳ Smʳ)	(Simon et al., 1983)
π3813	B462 Δ*thyA*::(*erm-pir-116*) (Ermʳ)	(Le Roux et al., 2007)

Plasmids

pGEM-T Easy	PCR products cloning vector, T-vector, Apʳ	Promega
pBluescript		
pBBR1MCS-5	broad host-range cloning vector, pBBR1, Gmʳ	(Kovach et al., 1995)
pDM4	Suicide plasmid *sacB* gene, R6K origin, Cmʳ	(Milton et al., 1996)
pEVS104	Conjugation helper plasmid, R6K origin, *RP4, oriT, tra, trb* and Kmʳ	(Septer et al., 2011)
pMMB208	A broad-host-range expression vector; Cmʳ *IncQ lacI*q Ptac; polylinker from M13mp19	(Morales et al., 1991)
pPROBE'-gfp[ASV]	Promoter probe vector, pBBR1, gfp[ASV], Kmʳ	(Miller et al., 2000) Addgene (40116)
pHN31	pDM4 with the Km resistance gene	This study
pHN32	pPROBE'-gfp[ASV] derivative, gfp[ASV] was replaced by lacIq-Ptac-polylinker from pMMB208	This study
pHN33	pHN32 derivative, the gentamicin resistance gene promoter from pBBR1MCS-5 was inserted.	This study

Table S2. Primers used in this study

Primer name	Sequence (5' to 3')
Mutagenesis	
For Δ*fttA*	
fttA-mut-*XhoI*-F	<u>CTCGAG</u>GAATGTGGGAAACACTCCACCTC
fttA-mut-*SmaI*-1	GTACATGCTTACGCCGGGCTAGAA<u>CCCGGG</u>AAACATCTAAACCCCCGGTAAATC
fttA-mut-*SmaI*-2	GATTTACCGGGGGTTTAGATGTTT<u>CCCGGG</u>TTCTAGCCCGGCGTAAGCATGTAC
fttA-mut-*SpeI*-R	<u>ACTAGT</u>GCGATGGACCACATCACAAACTGTG
For Δ*tnbA*	
tnbA-mut-*SacI*-F	<u>GAGCTC</u>TAAAGTGGCGCTACAGTGCATTTCAAC
tnbA-mut-*SmaI*-1	TTAAGTCTTCAGGCTGAGAGTGTAG<u>CCCCGGG</u>ATTTAATGCTTCGACGAGGCTGTTCATG
tnbA-mut-*SmaI*-2	ATGAACAGCCTCGTCGAAGCATTAAAT<u>CCCGGGG</u>CTACACTCTCAGCCTGAAGACTTAAC
tnbA-mut-*XhoI*-R	<u>CTCGAG</u>AATCTGGGTGCGCTCTAAATCGATTTC
For Δ*tnbF*	
tnbF-mut-*XhoI*-F	<u>CTCGAG</u>CTCTTCGATCAGTCGCGCAGG
tnbF-mut-*SmaI*-1	CTCACACGTTGGCGAGCTGCAC<u>CCCGGG</u>GAGTGTGTGCGATGTCATCAGATG
tnbF-mut-*SmaI*-2	CATCTGATGACATCGCACACACTC<u>CCCGGGG</u>GTGCAGCTCGCCAACGTGTGAG
tnbF-mut-*SpeI*-R	<u>ACTAGT</u>GAACCGTGTTCGAACAACTGGATAG
For Δ*tonB1ab*	
tonB1ab-mut-*XhoI*-F	<u>CTCGAG</u>AATCGGTTTAGATTCTTCACTCTTGTGC

tonB1ab-mut-*SmaI*-1	GCACTGCTGACTTGCAGAATTTA<u>CCCGGG</u>CATCAGCGGGACTCCTTGTCCTTAAG
tonB1ab-mut-*SmaI*-2	CTTAAGGACAAGGAGTCCCGCTGATG<u>CCCGGG</u>TAAATTCTGCAAGTCAGCAGTGC
tonB1ab-mut-*SpeI*-R	<u>ACTAGT</u>ACATCTACTCCTGCATAGTTAATCAC

For Δ*tonB2*

tonB2-mut-*XhoI*-F	<u>CTCGAG</u>TGTTTGGTACAACGTTGATTAACCG
tonB2-mut-*SmaI*-1	CATAAATATCACCCTGCCGCTA<u>CCCGGG</u>CATCGCTCACCTACCTGTTTTC
tonB2-mut-*SmaI*-2	GAAAACAGGTAGGTGAGCGATG<u>CCCGGG</u>TAGCGGCAGGGTGATATTTATG
tonB2-mut-*SpeI*-R	<u>ACTAGT</u>GCCATCTTCACTGCGATATTGATG

For Δ*tonB3*

tonB3-mut-*XhoI*-F	<u>CTCGAG</u>ACCATTCCTACCATGGCGGGCATG
tonB3-mut-*EcoRV*-1	CACGCATCGCTCTCCTCCTCGTTA<u>GATATC</u>CATCAATTACTGGCCTCCCTCGGCTGC
tonB3-mut-*EcoRV*-2	GCAGCCGAGGGAGGCCAGTAATTGATG<u>GATATC</u>TAACGAGGAGGAGAGCGATGCGT
tonB3-mut-*SpeI*-R	<u>ACTAGT</u>GCTCCCAATAGGTCGCCTTGTC

Complementation

For *fttA*

fttA-com-*KpnI*-F	<u>GGTACC</u>GATTTAATAGTATAACTTTATCGCTTATCG
fttA-com-*EcoRI*-R	<u>GAATTC</u>CTAGAAGCTATAAGTCGCACTCAAATAC

For *tonB1a*

tonB1a-com-*KpnI*-F	<u>GGTACC</u>GAATATCGCCCGTTTGAAGGCGCTTAAG
tonB1a-com-*EcoRI*-R	<u>GAATTC</u>TCAGCCTTCGAGGTTAAATACAAACG

For *tonB1b*

tonB1b-com-*KpnI*-F	<u>GGTACC</u>TTAACCGGCACTGTTGTACGTCAATCAC
tonB1b-com-*EcoRI*-R	<u>GAATTC</u>TTAGTCCTTCATCACAAAGGTGAGGC

For *tonB2*

tonB2-com-*XbaI*-F	<u>TCTAGA</u>GTGTTGGGGATGTCTCTGTCTC
tonB2-com-*SacI*-R	<u>GAGCTC</u>CTACTTGTCTTTGGCCATTTGGAAAG

For *tonB3*

tonB3-com-*KpnI*-F	<u>GGTACC</u>GGCGCGGAAAGTGTGTCTGTCGCAG
tonB3-com-*EcoRI*-R	<u>GAATTC</u>TTAGCGGCTCAGTTTAAATTCGATCG

qPCR

gyrB-qPCR-F	AGGCTTACCGGGGAAATTGG
gyrB-qPCR-R	CCTTTAAGCGGCAAGATCGC
fttA-qPCR-F	AGCAACACTGACCTGGAACC
fttA-qPCR-R	CTGCTCGCGGTAATCTTTGC
tnbA-qPCR-F	GAACGCACCATTCAAGGCTC
tnbA-qPCR-R	ATCGGATGCGAGAAACAGCA
tnbF-qPCR-F	ATCTGGAGAGCCACAACAGC
tnbF-qPCR-R	ATTTGGGTGAGCAGGGTGAG
tonB1a-qPCR-F	GCGACCTATCCCGCACTTTA
tonB1a-qPCR-R	AACCCGCTGTACAGTAGCAC
tonB1b-qPCR-F	GGAGGAGCCCAAGCTTGTTAT
tonB1b-qPCR-R	AGATGGGTGTCATCCGCTTG
tonB2-qPCR-F	TGCGGCACTGGTAACTCTTG
tonB2-qPCR-R	AATTTCCCGCTCAGGCATCA
tonB3-qPCR-F	GCCGAAGTTCTGGAAGCTGA
tonB3-qPCR-R	TGAGCCTTCGGCATACCATC

RNA-sequencing

T. turnerae T7901 was grown in non-aggregation SBM broth medium containing sucrose supplemented with 10 µM FAC (iron rich condition) or 0.1 µM FAC (iron limiting condition), and the expression of genes were compared by RNA-sequencing (RNA-seq). We avoided using an iron chelator to exclude any effects other than iron chelation caused by iron chelators. 2 biological replicates were used.

Illumina library construction using the Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero (Illumina) and sequencing using Novaseq 6000 with 150 bp paired-end runs was performed at the Huntsman Cancer Institute's High-Throughput Genomics Center at the University of Utah. All raw sequencing reads were deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA885807.

Quality control and preprocessing of FASTQ reads were performed using the fastp program (Chen et al., 2018). The processed reads were mapped on the chromosome of *T. turnerae* T7901 using HISAT2 (Kim et al., 2019) and the output was converted to bam files using SAMtools (Li et al., 2009). FeatureCounts (Liao et al., 2014) were used to count the reads on *T. turnerae* T7901 genes, and differential expression analysis was carried out using the EdgeR package (Robinson et al., 2010).

Table S3. RNA-seq analysis to understand iron regulation of iron transport genes

Turnerbactin biosynthesis and transport cluster

Locus tag	gene name or annotation	logFC	logCPM	FDR-adjusted p-value
TERTU_RS18025	*fttA*	8.55	12.09	2.94E-64
TERTU_RS18030	hypothetical protein	7.13	7.10	1.42E-66
TERTU_RS18035	hypothetical protein	7.39	7.23	9.20E-66
TERTU_RS18040	PepSY domain-containing protein	6.19	9.74	9.50E-46
TERTU_RS18045	*tnbC*	11.52	12.90	5.89E-93
TERTU_RS18050	*tnbE*	12.27	13.20	5.32E-98
TERTU_RS18055	*tnbB*	12.72	12.92	4.30E-98
TERTU_RS18060	*tnbA*	11.24	11.69	2.03E-88
TERTU_RS18065	efflux RND transporter periplasmic adaptor	10.83	11.60	1.20E-72
TERTU_RS18070	multidrug efflux RND transporter permease	6.9	12.44	1.84E-44
TERTU_RS18075	esterase	8.41	10.40	5.04E-65
TERTU_RS18080	MbtH domain protein	9.61	9.60	5.90E-76
TERTU_RS18085	*tnbF*	8.41	13.36	3.25E-60

TERTU_RS18090	*tnbS*	8.56	11.54	1.23E-62

TonB1 cluster

Locus tag	gene name or annotation	logFC	logCPM	FDR-adjusted p-value
TERTU_RS04350	transcriptional regulator	0.31	2.96	0.44
TERTU_RS04355	*tonB1a*	1.17	5.86	2.55E-03
TERTU_RS04360	*tonB1b*	0.33	3.93	0.49
TERTU_RS04365	*exbB1*	-0.59	3.12	0.1

TonB2 cluster

Locus tag	gene name or annotation	logFC	logCPM	FDR-adjusted p-value
TERTU_RS01630	*ttpB2*	-1.03	11.08	0.01
TERTU_RS01635	*ttpC2*	-0.81	11.59	0.05
TERTU_RS01640	*exbB2*	-0.74	10.02	0.09
TERTU_RS01645	*exbD2*	-0.76	9.50	0.09
TERTU_RS01650	*tonB2*	-0.51	9.78	0.26
TERTU_RS01655	*ttpD2*	-0.5	10.93	0.28

TonB3 cluster

Locus tag	gene name or annotation	logFC	logCPM	FDR-adjusted p-value
TERTU_RS08980	TonB-dependent receptor	-0.42	4.39	0.21

TERTU_RS08985	*ttpB3*	-1.61	1.06	3.69E-04
TERTU_RS08990	*ttpC3*	-0.81	2.55	0.03
TERTU_RS08995	*exbB3*	-0.84	1.82	0.03
TERTU_RS09000	*exbD3*	-0.6	1.65	0.16
TERTU_RS09005	*tonB3*	-1.12	2.54	2.20E-03
TERTU_RS09010	*ttpD3*	-0.6	4.11	0.07

Table S4 RNA-seq result to understand iron-regulation of genes potentially encoding TonB-dependent outer membrane receptor

Locus tag	logFC	logCPM	FDR-adjusted p-value
TERTU_RS00400	-0.50	5.33	0.16
TERTU_RS02235	-2.90	13.53	3.18E-08
TERTU_RS02920	0.02	9.41	0.97
TERTU_RS03510	-0.02	6.33	0.97
TERTU_RS05530	-1.72	5.88	1.14E-08
TERTU_RS05545	2.88	10.49	7.93E-13
TERTU_RS05620	-0.22	4.95	0.56
TERTU_RS06590	-0.85	5.08	0.01
TERTU_RS06645	-3.05	7.41	2.40E-14
TERTU_RS06655	0.03	12.84	0.95
TERTU_RS06660	-1.03	6.60	1.06E-03
TERTU_RS08920	0.27	5.13	0.49
TERTU_RS08980	-0.42	4.39	0.21
TERTU_RS09115	1.65	7.14	6.99E-04
TERTU_RS10285	-1.59	9.61	1.45E-03
TERTU_RS10395	-0.26	6.34	0.47
TERTU_RS11325	6.33	12.22	2.48E-44
TERTU_RS11935	-1.37	11.73	5.43E-04
TERTU_RS12735	-1.83	4.66	2.04E-05
TERTU_RS12820	-0.13	6.73	0.76
TERTU_RS14810	0.13	10.38	0.79
TERTU_RS14845	0.60	6.81	0.10
TERTU_RS14930	-0.15	7.46	0.72
TERTU_RS15340	-5.98	9.09	7.14E-19
TERTU_RS15625	-2.16	5.58	5.97E-06
TERTU_RS16015	-1.26	8.11	8.80E-04
TERTU_RS16355	0.44	7.52	0.24
TERTU_RS16735	1.66	8.63	1.45E-05
TERTU_RS17025	-1.76	8.63	2.25E-05
TERTU_RS17140	3.44	8.38	1.94E-19
TERTU_RS17895	4.09	9.96	2.41E-20
TERTU_RS18025	8.55	12.09	2.94E-64

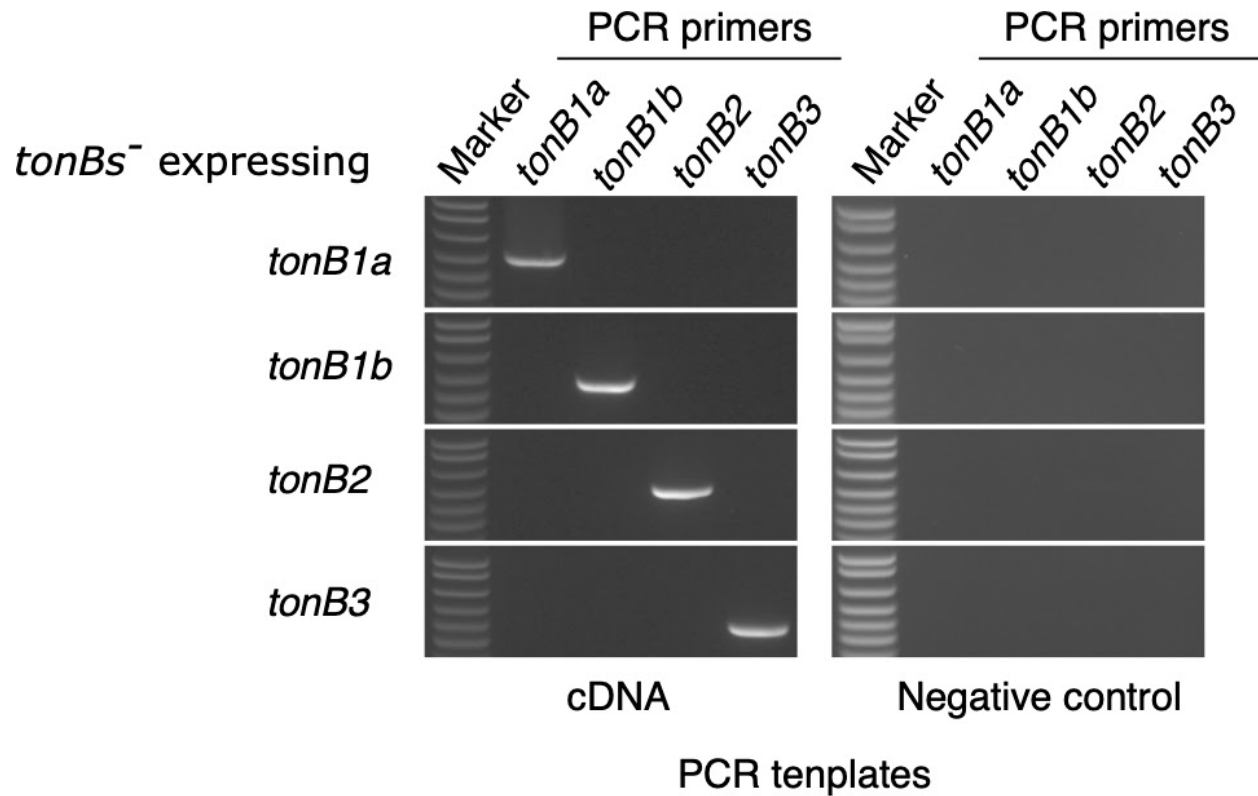|			
TERTU_RS18165	-2.09	5.48	7.17E-13
TERTU_RS18190	-1.39	4.11	1.30E-05
TERTU_RS18495	-2.11	7.59	9.63E-07
TERTU_RS19320	0.40	5.09	0.23
TERTU_RS20475	0.76	4.31	0.02
TERTU_RS20690	-0.72	12.24	0.09

Figure S1. RT-PCR to confirm the expression of TonB genes

Each TonB gene was cloned into the expression vector pHN45 and conjugated into the *T. turnerae* T7901 Δ*tonB1ab*Δ*tonB2*Δ*tonB3* (tonBs-) strain in which all *tonB* genes were deleted. The strains were grown in SBM medium containing sucrose (0.5%), FAC (10 μM) and Km (50 μg/ml) at 30°C until reaching the exponential phase (OD_{600} 0.2-0.3). Negative control, RT-reaction without RT enzyme; FAC, ferric ammonium citrate; pHN45, plasmid expression vector.

Fur titration assay

The fur titration assay (FURTA) was performed as described by (Stojiljkovic et al., 1994). DNA fragments to be tested were cloned into pBluescript II and transformed into E. coli H1717, and transformants were streaked on MacConkey agar plates supplemented with ammonium iron (II) sulfate (30µM) and Amp (100 µg/ml). Appearance of pink color (Lac+ phenotype, Fur-binding to cloned DNA fragments) around streaked *E. coli* was checked after overnight incubation at 37°C.
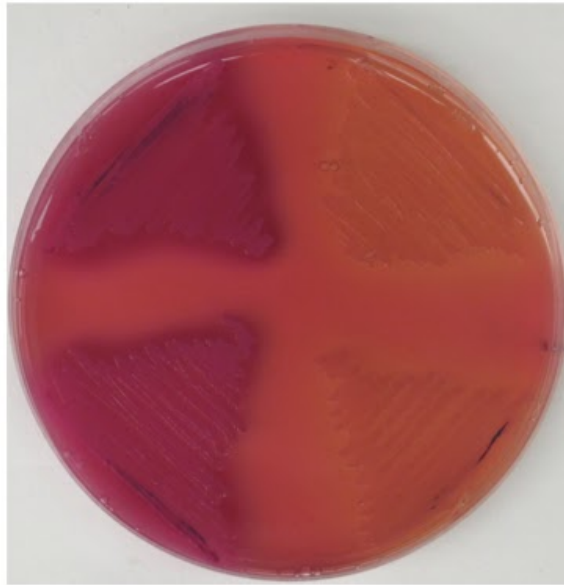
Figure S2. *E. coli* Fur can bind to the promoter regions of *fttA* and *tnbC*.

Binding of *E. coli* Fur to promoter regions of *fttA*, *tnbC*, and the region between *fttA* and *TERTU_RS18030* (negative control) was tested by Fur titration assay (Stojiljkovic et al., 1994). *E. coli* H1717 containing plasmids harboring each fragment were streaked on MacConkey agar plates, and the presence of pink color (Lac+ phenotype) around streaked bacteria was evaluated after overnight incubation at 37°C. pBlue, pBluescript II; *fttA* prom, *fttA* promoter region; *tnbC* prom, *tnbC* promoter region; *fttA-RS18030*, the intergenic region between *fttA* and *TERTU_RS18030*.

References

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34**,** i884-i890.

Distel, D.L., Morrill, W., Maclaren-Toussaint, N., Franks, D., and Waterbury, J. (2002). Teredinibacter turnerae gen. nov., sp. nov., a dinitrogen-fixing, cellulolytic, endosymbiotic gamma-proteobacterium isolated from the gills of wood-boring molluscs (Bivalvia: Teredinidae). *Int J Syst Evol Microbiol* 52**,** 2261-2269.

Kim, D., Paggi, J.M., Park, C., Bennett, C., and Salzberg, S.L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol* 37**,** 907-915.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., 2nd, and Peterson, K.M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166**,** 175-176.

Le Roux, F., Binesse, J., Saulnier, D., and Mazel, D. (2007). Construction of a Vibrio splendidus mutant lacking the metalloprotease gene vsm by use of a novel counterselectable suicide vector. *Appl Environ Microbiol* 73**,** 777-784.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., and Genome Project Data Processing, S. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25**,** 2078-2079.

Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30**,** 923-930.

Miller, W.G., Leveau, J.H., and Lindow, S.E. (2000). Improved gfp and inaZ broad-host-range promoter-probe vectors. *Mol Plant Microbe Interact* 13**,** 1243-1250.

Milton, D.L., O'toole, R., Horstedt, P., and Wolf-Watz, H. (1996). Flagellin A is essential for the virulence of Vibrio anguillarum. *J Bacteriol* 178**,** 1310-1319.

Morales, V.M., Backman, A., and Bagdasarian, M. (1991). A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. *Gene* 97**,** 39-47.

Robinson, M.D., Mccarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26**,** 139-140.

Septer, A.N., Wang, Y., Ruby, E.G., Stabb, E.V., and Dunn, A.K. (2011). The haem-uptake gene cluster in Vibrio fischeri is regulated by Fur and contributes to symbiotic colonization. *Environ Microbiol* 13**,** 2855-2864.

Simon, R., Priefer, U., and Pühler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in gram negative bacteria. *Bio/Technology* 1**,** 784-791.

Stojiljkovic, I., Baumler, A.J., and Hantke, K. (1994). Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated Escherichia coli genes by a fur titration assay. *J Mol Biol* 236**,** 531-545.