1   **Thiamine pyrophosphate riboswitches in *Bacteroides* species regulate transcription or**

2   **translation of thiamine transport and biosynthesis genes**

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11   Short title: Regulation by TPP Riboswitches in *Bacteroides*

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18 **Abstract**

19   Thiamine (vitamin B_1) and its phosphorylated precursors are necessary for

20   decarboxylation reactions required in carbohydrate and branched chain amino acid metabolism.

21   Due to its critical roles in central metabolism, thiamine is essential for human and animal hosts

22   and their resident gut microbes. However, little is known about how thiamine availability shapes

23   the composition of gut microbial communities and the physiology of individual species within

24   those communities. Our previous work has implicated both thiamine biosynthesis and transport

25   activities in the fitness of *Bacteroides* species. To better understand thiamine-dependent gene

26   regulation in *Bacteroides*, we examined thiamine biosynthesis and transport genes in three

27   representative species: *Bacteroides thetaiotaomicron*, *Bacteroides uniformis*, and *Bacteroides*

28   *vulgatus*. All three species possess thiamine biosynthetic operons controlled by highly

29   conserved *cis*-acting thiamine pyrophosphate (TPP) riboswitches. *B. thetaiotaomicron* and *B.*

30   *uniformis* have additional TPP riboswitch-controlled operons encoding thiamine transport

31   functions. Transcriptome analyses showed that each *Bacteroides* species had a distinct

32   transcriptional response to exogenous thiamine. Analysis of transcript levels and translational

33   fusions demonstrated that in *B. thetaiotaomicron,* the TPP riboswitch upstream of biosynthesis

34   genes acts at the level of transcription, while TPP riboswitches upstream of transport operons

35   work at the level of translation. In *B. uniformis* and *B. vulgatus,* TPP riboswitches work at the

36   transcriptional level to control downstream operons. The varying responses to exogenous

37   thiamine and use of varied regulatory mechanisms may play an important role in niche

38   establishment by the Bacteroidetes in the complex and constantly shifting gut environment.

39

40   **Importance**

41   *Bacteroides* species are important and abundant members of human gut microbiome

42   communities. Their activities in the gut are influenced by constant changes in nutrient

43   availability. In this study, we investigated the genetic basis of thiamine (Vitamin B_1) uptake and

44   biosynthesis in three representative *Bacteroides* species. We found species-specific differences

45   in the response to exogenous thiamine, and distinct mechanisms for regulation of uptake and

46   biosynthesis gene expression. Our work implies that gut *Bacteroides* have evolved distinct

47   strategies for making or acquiring an essential nutrient. These mechanisms may play an

48   important role in the success of *Bacteroides* in establishing a niche within complex gut

49   microbiome communities.

50

51 **Introduction**

52   Thiamine pyrophosphate (TPP) is the active cofactor form of thiamine (Vitamin B_1).

53   Thiamine is required by all living organisms to perform a variety of metabolic activities.

54   Thiamine-requiring pathways include central metabolism, branched chain amino acid

55   biosynthesis, and nucleotide synthesis (1-4). Humans primarily acquire thiamine from their diet

56   and convert it to the cofactor form, TPP (5, 6). In addition to dietary thiamine, there is some

57   evidence that microbial communities in the gastrointestinal tract can provide thiamine to the host

58   (7, 8). This thiamine may be supplied by resident gut microbes that possess the ability to

59   synthesize thiamine and TPP *de novo* (9,10). A recent study suggested that certain pathogenic

60   microbes can inhibit thiamine uptake by host intestinal epithelial cells (11). In addition, thiamine

61   availability has been correlated with differences in composition of gut microbial communities

62   (12). Thiamine excess and thiamine deficiency of the human host have been linked to different

63   diseases, including Crohn's Disease, Irritable Bowel Disease, and Dementia, which are also

64   associated with altered gut microbiome structure and function (6,13,14). While these studies

65   reveal interesting correlations that suggest a role for thiamine at the host-microbe interface, very

66   little is known about how dominant members of the gut microbiota sense and respond to

67   thiamine availability.

68   Work to characterize and understand thiamine biosynthesis and transport has primarily

69   been done in model organisms including *Escherichia coli, Salmonella enterica,* and *Bacillus*

70   *subtilis* (10, 15-18). Thiamine biosynthesis is carried out by a complex suite of enzymes through

71   a conserved bifurcated pathway. The first key intermediates in TPP synthesis are 4-amino-2-

72   methyl-5-diphosphomethylpyrimidine (HMP-PP) and 2-(2-carboxy-4-methylthiazol- 5-yl) ethyl

73   phosphate (cTHz-P). HMP-PP and cTHz-P are then condensed into thiamine monophosphate

74   (TMP) and finally phosphorylated to make the active cofactor TPP (15). Thiamine biosynthesis

75   activities are found in bacteria, yeast, and plants. Transport proteins specific for thiamine,

76   thiamine monophosphate (TMP), TPP, and other thiamine precursors are broadly distributed

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77   across all three domains of life (10, 19-23). Almost without exception, the genes and operons

78   encoding these diverse thiamine transport and biosynthesis pathways are regulated by highly

79   conserved TPP riboswitches.

80   Riboswitches are highly conserved *cis*-encoded and *cis*-acting regulatory RNAs found in

81   the untranslated regions of messenger RNAs. Riboswitches bind small molecules, changing

82   mRNA secondary structure and controlling expression outcomes at the level of transcription

83   elongation, translation initiation, or RNA splicing (24, 25). TPP riboswitches are unique in that

84   they are the only riboswitch found across all three domains of life (26). TPP riboswitches are so

85   highly conserved, they are now used to identify thiamine biosynthesis and transport genes in

86   diverse organisms (9). In bacteria, TPP riboswitches regulate biosynthesis and transport genes

87   at either the transcriptional or translational level (27). TPP-bound forms of riboswitches that

88   control transcription of downstream genes promote formation of a stem-loop that causes

89   premature transcription termination. TPP riboswitches that act at the level of translation promote

90   formation of a secondary structure that blocks ribosome binding when TPP is bound (28). TPP

91   riboswitches that act at the level of transcription have been found primarily in Gram-positive

92   bacteria, whereas Gram-negative bacteria tend to have TPP riboswitches that act at the level of

93   translation (29). It has been noted that TPP riboswitches in *E. coli* that repress translation (when

94   TPP is bound) also reduce mRNA stability, resulting in reduced steady-state levels of the

95   transcript (30). While TPP riboswitch structures and functions have been extensively studied in

96   the model organisms *E. coli* and *B. subtilis*, there has been little characterization of these

97   conserved regulatory elements in other microbes, including those belonging to the phylum

98   Bacteroidetes.

99   Computational analyses predicted that TPP riboswitches are present and regulate

100   putative thiamine biosynthesis and transport pathways across the Bacteroidetes (9, 22). TPP

101   riboswitch-controlled genes in *Bacteroides thetaiotaomicron,* a prominent member of the

102   Bacteroidetes, have recently been characterized (10, 20). These studies have shown that both

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103   thiamine transport and biosynthesis are critical to the fitness of *B. thetaiotaomicron* under

104   thiamine-limiting growth conditions. In this study, we build on previous work to investigate the

105   global gene expression response of three representative *Bacteroides* species to exogenous

106   thiamine and to determine the mechanisms of TPP riboswitch-dependent regulation of thiamine

107   biosynthesis and transport genes. We found that each of the three species we studied had a

108   distinct transcriptional response to exogenous thiamine. Investigation of the mechanisms of TPP

109   riboswitch-mediated regulation of downstream genes and operons revealed that *B.*

110   *thetaiotaomicron* uses both transcriptional- and translational-acting riboswitches, whereas

111   *Bacteroides uniformis* and *Bacteroides vulgatus* use TPP riboswitches that act at the level of

112   transcription. Identification of TPP riboswitches across the Bacteroidetes revealed a few

113   different configurations of TPP riboswitch-controlled operons and suggested that both

114   transcriptional and translational mechanistic classes are widely distributed.

115
116   **Results**

117   *Global transcriptional responses to exogenous thiamine vary between* Bacteroides *species.*

118   Our previous study identified putative thiamine biosynthesis and transport genes in 114

119   *Bacteroides* genomes (10). We found biosynthesis and transport genes in 107 of 114 genomes,

120   and a few genomes that appeared to contain either biosynthesis (2 of 114) or transport (5 of

121   114) genes. To further understand the diversity of thiamine biosynthesis and transport functions

122   and regulation in *Bacteroides*, we chose three representative species to further characterize in

123   this study. In *B. thetaiotaomicron*, we identified and characterized three gene clusters (Fig. 1A,

124   1C, 1E) involved in thiamine biosynthesis (*thiSEGCHF$_{Bt}$-tenI$_{Bt}$*), outer membrane thiamine

125   transport (*OMthi$_{Bt}$*) and cytoplasmic membrane transport (*pnuT$_{Bt}$-tnr3$_{Bt}$*) in a previous study (10).

126   Each of these three gene clusters is preceded by a putative TPP riboswitch (Fig. 1A). In *B.*

127   *uniformis,* we identified two putative TPP riboswitch-controlled gene clusters: for biosynthesis

128   (*thiSEGCHF$_{Bun}$-tenI$_{Bun}$*) and transport (*OMthi$_{Bun}$-pnuT$_{Bun}$-tnr3$_{Bun}$*) (Fig. 1C). *B. vulgatus* has only a

129   single putative TPP riboswitch dependent operon for thiamine biosynthesis (*thiSEGCHF$_{Bv}$-*

130   *tenI$_{Bv}$*) and apparently lacks thiamine transport genes (Fig. 1E).

131   Our previous study showed that *B. thetaiotaomicron* differentially expresses genes

132   comprising a number of pathways, including thiamine biosynthesis and transport, in the

133   presence versus absence of exogenous thiamine (10). To determine if the transcriptional

134   response to thiamine limitation is consistent across *Bacteroides* species we analyzed

135   transcriptomes of *B. uniformis* and *B. vulgatus* in minimal medium with and without exogenous

136   thiamine (as described in Materials and Methods and (10)). A global view of differential gene

137   expression in the absence versus presence of exogenous thiamine is shown for *B.*

138   *thetaiotaomicron* (Fig. 1B and (10)), *B. uniformis* (Fig. 1D) and *B. vulgatus* (Fig. 1F). Whole

139   transcriptome responses to exogenous thiamine were markedly different among these three

140   species. *B. thetaiotaomicron* had 151 genes significantly differentially expressed by ≥ 2-fold in

141   the presence of exogenous thiamine compared to its absence (Fig. 1B). Genes that showed

142   reduced expression in the presence vs. absence of exogenous thiamine in *B. thetaiotaomicron*

143   encode functions belonging to a few pathways, including thiamine biosynthesis, amino acid

144   biosynthesis, TCA cycle enzymes, purine, and pyruvate metabolism (Table S1 and 10). In

145   contrast, minimal transcriptome responses to exogenous thiamine were observed in *B.*

146   *uniformis* (Fig. 1D) and *B. vulgatus* (Fig. 1F). *B. uniformis* responded to exogenous thiamine

147   with reduced expression of the genes in the TPP riboswitch-controlled thiamine biosynthesis

148   cluster (*thiSEGCHF$_{Bun}$-tenI$_{Bun}$*, Fig. 1C, 1D). There were no significant differences in the gene

149   expression profile of *B. vulgatus* in the absence vs. presence of exogenous thiamine (Fig. 1F).

150   Our previous study revealed that *B. vulgatus* lacks an *OMthi* homolog found in many other

151   *Bacteroides* species (10), suggesting that *B. vulgatus* may not be able to recognize or take up

152   exogenous thiamine. In addition, while *B. vulgatus* does have a *pnuT* homolog, this gene is not

153   preceded by a TPP riboswitch and is not clustered with other putative thiamine transport or

154   biosynthesis genes, suggesting that this PnuT-like protein may have a different substrate

155   specificity compared to the *B. thetaiotaomicron* and *B. uniformis* PnuT proteins (20, 31).

156   Together, these data suggest that *B. vulgatus* does not respond transcriptionally to exogenous

157   thiamine because it is not recognized or transported.

158   TPP riboswitches have been found to control either transcription elongation or

159   translation initiation, depending on the organism (27). We noted that in *B. thetaiotaomicron* and

160   *B. uniformis,* TPP riboswitch-controlled operons for biosynthesis and transport had different

161   responses to exogenous thiamine at the level of mRNA abundance based on RNA-seq

162   experiments. We hypothesized that this might reflect differences in TPP riboswitch-mediated

163   mechanisms of regulation of these different operons. In *B. thetaiotaomicron,* only the

164   biosynthesis operon mRNAs were differentially regulated in response to exogenous thiamine

165   (Fig. 1A, (10)). The abundance of $OMthi_{Bt}$ and $pnuT_{Bt}$-$tnr3_{Bt}$ mRNAs encoding transport

166   functions in *B. thetaiotaomicron* was not significantly different in the presence or absence of

167   exogenous thiamine (Fig. 1A, (10)), suggesting that the TPP riboswitches controlling *B.*

168   *thetaiotaomicron* thiamine transport genes might act at the level of translation. The levels of

169   TPP riboswitch-controlled biosynthesis and transport operon mRNAs in *B. uniformis* were

170   reduced in the presence of exogenous thiamine (Fig. 1C). To confirm the results of RNA-seq

171   with respect to thiamine-induced changes in mRNA levels, RT-qPCR was performed. Primers

172   were designed to measure levels of biosynthesis (*thiC*), outer membrane TonB-dependent

173   transporter (*OMthi*), and inner membrane transporter (*pnuT*) mRNAs in each organism. The

174   mRNA abundance trends observed in all three organisms in RNA-seq were replicated in RT-

175   qPCR experiments. In *B. thetaiotaomicron*, $thiC_{Bt}$ mRNA levels were 60-fold higher in cells

176   grown without thiamine compared to cells grown in the presence of 10 µM thiamine (Fig. 2A).

177   The abundance of $OMthi_{Bt}$ and $pnuT_{Bt}$ mRNAs was equivalent in cells grown with and without

178   thiamine. In *B. uniformis,* $thiC_{Bun}$ mRNA levels were 2-fold higher in cells grown without thiamine

179   compared to cells grown with thiamine (Fig. 2B). In contrast to *B. thetaiotaomicron*, *B. uniformis*

180   transporter mRNA ($OMthi_{Bun}$ $pnuT_{Bun}$) levels also responded to exogenous thiamine with ~3-fold

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181   higher levels in cells grown without thiamine (Fig. 2B). Transcript levels of the *B. vulgatus* TPP

182   riboswitch-controlled biosynthesis operon did not respond to exogenous thiamine (Fig. 2C), also

183   consistent with RNA-seq results. These data suggest that in *Bacteroides* organisms that have

184   thiamine transport functions, exogenous thiamine affects transcript levels of thiamine

185   biosynthesis genes. The transcriptional response to thiamine varied between species for

186   mRNAs encoding transport functions.

187
188   *Translational regulation of TPP-controlled operons in* Bacteroides

189   We next investigated if TPP riboswitch-controlled operons that did not show a

190   transcriptional response to exogenous thiamine were instead regulated at the level of

191   translation. We constructed nanoluciferase translational fusions to the start codon of the first

192   gene in TPP riboswitch-controlled operons. All nanoluciferase (NanoLuc) fusions are controlled

193   by native promoters and *cis*-acting TPP riboswitches. It is important to note that these

194   translational fusions would capture thiamine-dependent transcriptional regulation caused by

195   riboswitch-mediated premature transcription termination and translational regulation, *e.g.,*

196   riboswitch-mediated changes in ribosome binding site accessibility. Fusions for the three

197   predicted TPP riboswitches (numbered as indicated in Fig. 1A) in *B. thetaiotaomicron* are called

198   pBtTPP1, pBtTPP2 and pBtTPP3 (Fig. 3A). *B. uniformis* fusions were pBunTPP1 and

199   pBunTPP2 (Fig. 3B), and the *B. vulgatus* fusion was called pBvTPP1 (Fig. 3C). Strains

200   containing each fusion were grown in media containing a range of exogenous thiamine

201   concentrations (from 0 to 10,000 nM). Promoterless NanoLuc controls were tested in each

202   strain background without and with 10,000 nM thiamine. These controls showed that thiamine

203   did not significantly alter luminescence (RLU/OD$_{630}$), as indicated by dashed lines in Fig. 3A-D.

204   In *B. thetaiotaomicron,* the BtTPP1 reporter fusion was repressed as exogenous

205   thiamine concentrations increased, with statistically significant differences beginning at 10 nM

206   thiamine (Fig. 3A). This result, combined with the reduced levels of *thiSEGCHF$_{Bt}$-tenI$_{Bt}$* mRNA

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207   observed in RNA-seq (Fig. 1B) and RT-qPCR (Fig. 2A), suggests that BtTPP1 riboswitch acts at

208   the level of transcription. The *B. thetaiotaomicron* BtTPP2 and BtTPP3 riboswitches control

209   thiamine transport *OMthi$_{Bt}$* and *pnuT$_{Bt}$-tnr3$_{Bt}$* genes, and the levels of these mRNAs were not

210   affected by exogenous thiamine (Figs. 1B and 2A). In contrast, the BtTPP2 and BtTPP3

211   reporters were repressed in the presence of exogenous thiamine, starting at 100 nM (Fig. 3A).

212   These data suggest that the *B. thetaiotaomicron* riboswitches controlling transport operons

213   regulate at the level of translation.

214   The reporters fused to *B. uniformis* riboswitches controlling biosynthesis (BunTPP1) and

215   transport (BunTPP2) operons followed a pattern similar to that observed in *B. thetaiotaomicron.*

216   The biosynthesis riboswitch reporter BunTPP1 was repressed by concentrations of exogenous

217   thiamine at or above 10 nM, while the transport riboswitch reporter BunTPP2 required

218   concentrations of 100 nM or greater for repression (Fig. 3B). These data, along with RNA-seq

219   (Table S1) and RT-qPCR (Fig. 2B) are consistent with the model that both TPP riboswitches in

220   *B. uniformis* act at the level of transcription.

221   In *B. vulgatus,* the riboswitch reporter controlling biosynthesis genes (BvTPP1) did not

222   respond to exogenous thiamine (Fig. 3C). However, since it appears that *B. vulgatus* lacks a

223   thiamine transport system, we reasoned that the lack of a response could be due to failure to

224   take up and accumulate the thiamine provided exogenously. To test whether the BvTPP1

225   riboswitch could respond to thiamine in an organism competent for thiamine transport, we

226   moved BvTPP1-NanoLuc into *B. thetaiotaomicron* (*Bt* pBvTPP1-NanoLuc, Fig. 3D). In a *B.*

227   *thetaiotaomicron* background, the *B. vulgatus* BvTPP1 biosynthesis riboswitch was repressed at

228   concentrations at or above 10 nM exogenous thiamine (Fig. 3D). This response is similar to

229   both the *B. thetaiotaomicron* and *B. uniformis* thiamine biosynthesis regulating TPP riboswitches

230   (Fig. 3A, B). The response to exogenous thiamine observed for the *B. vulgatus* TPP1 riboswitch

231   reporter in *B. thetaiotaomicron* shows that the biosynthesis regulating riboswitch of *B. vulgatus*

232   is functional. To determine whether the *B. vulgatus* TPP1 riboswitch controls transcription or

233   translation of biosynthesis genes, we performed RT-qPCR on *B. thetaiotaomicron* strains

234   carrying reporter fusions to detect changes in NanoLuc fusion mRNAs in the absence and

235   presence of thiamine (Fig. 3E). As controls we also monitored levels of fusion mRNAs for the *B.*

236   *thetaiotaomicron* transcriptional riboswitch (BtTPP1) and the translational riboswitches (BtTPP2

237   and BtTPP3). The results for controls were as expected – levels of the biosynthesis BtTPP1

238   reporter mRNA were reduced in the presence of thiamine, and levels of the transporter BtTPP2

239   and BtTPP3 fusion mRNAs were unchanged. The levels of *B. vulgatus* biosynthesis BvTPP1

240   reporter mRNA were also reduced in response to exogenous thiamine in the *B.*

241   *thetaiotaomicron* strain background (Fig. 3E) suggesting that the *B. vulgatus* TPP riboswitch

242   acts at the level of transcription.

243   Collectively, these data suggest *Bacteroides* species utilize riboswitches that act at

244   either the transcriptional or the translational level. The riboswitches controlling biosynthesis

245   operons are more sensitive than transport operon riboswitches, suggesting a hierarchical

246   response to thiamine concentrations in the range of 10 to 100 nM.

247
248   *Distances of TPP Riboswitches Correspond to Regulatory Mechanism*

249   Our results so far suggest that several TPP riboswitches – BtTPP1, BunTPP1,

250   BunTPP2, and BvTPP1 – in *B. thetaiotaomicron*, *B. uniformis*, and *B. vulgatus* regulate

251   downstream gene expression at the level of transcription. In contrast, BtTPP2 and BtTPP3

252   regulation was only observed at the level of translation. We observed that the transcriptionally

253   acting riboswitches in *B. thetaiotaomicron, B. uniformis, and B. vulgatus* were located further

254   upstream from the first gene in the operon compared to the translational-acting riboswitches in

255   *B. thetaiotaomicron.* The differences in distance between the TPP riboswitch and the

256   downstream gene that correlate with regulatory mechanism are consistent with previously

257   characterized TPP riboswitches in model organisms (25, 29, 30). Transcriptional-acting

258   riboswitches commonly found in Gram-positive bacteria are typically located >40-nt upstream of

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259   the start codon of the adjacent gene, whereas translational-acting riboswitches often found in

260   Gram-negative bacteria are usually <30-nt from the downstream gene. These trends that are

261   consistent with our observations in *Bacteroides* led us look more globally at the distance

262   between TPP riboswitches and downstream genes across the Bacteroidetes.

263   We analyzed 219 TPP riboswitches in 114 Bacteroidetes genomes. The riboswitches

264   were grouped and categorized according to the genes that they regulate (Fig 4A, Table 2).

265   Among the 219 riboswitches identified, 122 were predicted to regulate thiamine biosynthetic

266   operons. *Bacteroides* species have biosynthetic operons in which critical genes for thiazole

267   synthesis, pyrimidine synthesis, and thiamine condensation are in the same TPP riboswitch-

268   controlled operon (Fig. 4A). More distantly related Bacteroidetes, e.g., *Parabacteroides* and

269   *Paraprevotella* species appear to have split biosynthesis operons, with one TPP-controlled gene

270   cluster for thiazole synthesis and a second for HMP synthesis and TMP condensation (Fig. 4A).

271   A total of 97 TPP riboswitches were found upstream of predicted thiamine transport genes,

272   primarily operons with *OMthi* (85 of 97) or *pnuT* (12 of 97) as the riboswitch-proximal gene (Fig.

273   4A, Table 2). In contrast to the diversity observed among TPP riboswitch controlled thiamine

274   biosynthesis operons; thiamine transport operon organization is highly conserved. TPP

275   riboswitches regulate both the inner and outer membrane transporters in addition to a thiamine

276   pyrophosphokinase (*tnr3*) (Fig. 4A).

277   For the transcriptional-acting TPP riboswitches in *B. uniformis, B. vulgatus,* and *B.*

278   *thetaiotaomicron* (biosynthetic operon only), we observed a >50-nt distance between the TPP

279   riboswitch and the start codon of the first gene in the operon. In contrast, for the *B.*

280   *thetaiotaomicron* thiamine transport operons, the TPP riboswitch was <5-nt from the first gene in

281   each operon. We next analyzed the distances between TPP riboswitches and downstream

282   genes more broadly across the Bacteroidetes (Table 2). While there is a range, the average

283   distance between TPP riboswitches and downstream thiamine biosynthesis genes is 60 nt (± 34

284   nt). The average distance between TPP riboswitches and downstream thiamine transport genes

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285   is shorter at 21 nt (± 20 nt) (Table 2). We plotted the number of occurrences of riboswitches at

286   specific distances from downstream genes, classified by the predicted function of the

287   downstream operon (Fig. 4B). This analysis illustrated the overall pattern we observed, where

288   TPP riboswitches upstream of biosynthesis genes were located further from the start codon and

289   TPP riboswitches upstream of transporter genes were located closer to the start codon. Based

290   on our experimental data from *B. thetaiotaomicron, B. uniformis,* and *B. vulgatus* and the

291   observed TPP riboswitch distances across other gut Bacteroidetes, we predict that the TPP

292   riboswitches located <20 nt from the start codon regulate at the level of translation while

293   riboswitches >40 nt from the start codon regulate at the level of transcription.

294
295   **Discussion**

296   Thiamine plays an essential role in both host and microbial metabolism, so thiamine

297   biosynthesis and transport may play a key role in modulating the dynamics of host-microbe

298   interactions. It is thought that most of the thiamine in the gastrointestinal tract is derived from the

299   host's diet (5, 23), and that microbial community composition may be influenced by competition

300   for this key nutrient (10). In this study, we characterized variable TPP riboswitch-dependent

301   regulation of thiamine biosynthesis and transport genes in three representative *Bacteroides*

302   species. In all three organisms, the thiamine biosynthesis genes were controlled at the level of

303   transcription by a TPP riboswitch located ~50-nt upstream from *thiS*, the first gene in the

304   biosynthesis operon. In *B. thetaiotaomicron*, the thiamine transport genes were controlled at the

305   level of translation, by TPP riboswitches encoded immediately upstream of the start codons of

306   transport genes. When we examined gut Bacteroidetes more broadly, we found that the

307   distances between TPP riboswitches and downstream start codons were consistently correlated

308   with the functional category, with longer distances for biosynthesis genes and shorter distances

309   for transport genes. We postulate that in the Bacteroidetes, TPP riboswitches upstream of

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310   biosynthesis genes commonly control expression at the level of transcription elongation

311   whereas riboswitches upstream of transport genes frequently control translation initiation.

312   Historically, it was thought that mechanisms of regulation by TPP-dependent

313   riboswitches were divided between Gram-negative bacteria, where regulation of translation was

314   common, and Gram-positive bacteria, where transcription attenuation was typical (29, 32, 33).

315   Recent studies have demonstrated that these distinctions are not so clear. For example, the

316   *lysC* riboswitch in *E. coli* is a dual-acting riboswitch. Ligand binding promotes both translational

317   repression and RNase E-dependent cleavage and subsequent mRNA decay (30). Chauvier, *et*

318   al., recently found a role for transcriptional pausing in modulation of TPP binding and

319   subsequent regulatory events for the *E. coli thiC* riboswitch (34, 35). Pausing of the transcription

320   complex at a regulatory site within the *thiC* riboswitch inhibits TPP binding and allows

321   transcription elongation and translation. If TPP binds co-transcriptionally, translation is inhibited

322   and Rho-dependent termination occurs. Our results further emphasize that diverse organisms

323   use both transcriptional and translational strategies for regulation mediated by TPP-dependent

324   riboswitches.

325   Trends in distances between TPP riboswitches and downstream genes in *B.*

326   *thetaiotaomicron*, *B. uniformis* and *B. vulgatus* suggest that riboswitches located further

327   upstream from the adjacent start codon control downstream gene expression at the level of

328   transcription elongation. These more distant riboswitches were found upstream of thiamine

329   biosynthesis genes in all three organisms and thiamine transport genes in *B. uniformis,* and all

330   promoted a thiamine-dependent reduction in the respective mRNAs. It is possible that one or

331   more of these riboswitches could act at both transcriptional and translational levels, or that

332   translational regulation could be coupled to nuclease-dependent degradation. The riboswitches

333   upstream of thiamine transport genes in *B. thetaiotaomicron* were directly adjacent to the

334   downstream start codons, and these riboswitches clearly regulated translation in response to

335   thiamine, but had no effect on transcript levels. The molecular details of TPP riboswitch-

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336   dependent regulation in *Bacteroides* species will require additional work. In these organisms,

337   much remains to be discovered even in terms of basic mechanisms of regulation of

338   transcription, translation, and mRNA processing and decay.

339   Our translational fusions revealed that TPP riboswitches regulating biosynthesis genes

340   were responsive to thiamine at lower concentrations compared to riboswitches controlling

341   transport genes. Biosynthesis genes were significantly downregulated in the presence of 10 nM

342   thiamine, while transport genes responded only to concentrations at or above 100 nM. These

343   concentrations are similar to the concentrations at which *E. coli* TPP riboswitches regulate

344   cognate genes (33), suggesting that internal thiamine requirements may be similar across gut

345   microbes. In addition, the 10 to 100 nM thiamine concentration range corresponds to the

346   expected concentration of thiamine in the gut (20 to 2000 nM) (5). These data suggest that TPP

347   riboswitch affinities among the Bacteroidetes and other gut bacteria may have evolved to

348   operate at levels common to the human intestinal tract and to build a hierarchy of regulation

349   between thiamine biosynthesis and transport to deal with rapidly changing nutrient availability in

350   the gut. Such regulatory systems are not uncommon in the Bacteroidetes as a similar

351   hierarchical regulatory phenomenon was observed for vitamin B_{12} riboswitches which respond

352   to different concentrations of B_{12} to differentially control when transporters of varying affinities

353   are expressed (36). It is worth noting that despite the similarities between thiamine and B_{12}

354   riboswitches in hierarchical responses to concentrations of the cognate vitamin, the B_{12}

355   riboswitches found in Bacteroidetes have only been observed to act at the level of translation

356   (37).

357   We inspected the publicly available genomes to identify TPP riboswitches in 114

358   Bacteroidetes genomes and found that TPP riboswitches were commonly found upstream of

359   putative thiamine biosynthetic and transport operons. These predicted riboswitches appear to

360   follow the distance trends observed in *B. thetaiotaomicron, B. uniformis,* and *B. vulgatus.* If

361   distances between the TPP riboswitch and downstream start codon are indicative of regulatory

15

362   mechanism, as suggested by our results, then Bacteroidetes commonly use both transcriptional

363   and translational acting TPP riboswitches to maintain internal thiamine homeostasis in the gut.

364   While regulatory thresholds of Bacteroidetes TPP riboswitches may be similar across species

365   and genera, global responses to thiamine are distinct. We have shown that *B. thetaiotaomicron*

366   has a large suite of genes that are differentially regulated in the presence of exogenous

367   thiamine, while closely related *B. vulgatus* and *B. uniformis* have no or a much narrower

368   response. Such regulon differences have been observed in *E. coli* and *Salmonella enterica*

369   where thiamine metabolism in the absence of thiamine show strikingly different global effects. In

370   *E. coli,* branched-chain amino acid catabolism is involved while *S. enterica* leverages purine

371   metabolism to produce precursors for thiamine synthesis (16-18).

372   Bacteroidetes TPP riboswitch structures are highly conserved across the species

373   investigated we have investigated here, but we have shown that at least *B. thetaiotaomicron*

374   utilizes both transcriptional and translational acting TPP riboswitches. While a single organism

375   using different mechanisms for TPP riboswitch-dependent regulation is rare, it is not unheard of

376   for a single riboswitch to transcriptionally regulate genes for biosynthesis of its ligand while

377   regulating its transporter at the level of translation (25, 30). While the structure of the adaptor

378   domain that binds TPP is highly conserved, the expression platform evolves much more quickly

379   and likely has the largest impact on the mechanism of regulation mediated by each riboswitch

380   (38). When riboswitches first evolved in the RNA world, it is believed that transcriptional control

381   came first and allowed riboswitches to control the function of ribozymes (39). The regulation of

382   translation likely evolved later and was retained because it is advantageous in some cases to

383   have a reversible form of regulation to respond to rapidly changing environmental

384   concentrations of the riboswitch ligand (38, 40). In the context of Bacteroidetes in the gut it may

385   be beneficial to have transporters regulated at the level of translation as it is a reversible and

386   fast-acting mechanism of regulation that could respond to rapidly changing thiamine

387   concentrations in the gut. Conversely, the tighter control of thiamine biosynthesis in the

388   Bacteroidetes via transcriptional regulation may help save the energy of producing multiple

389   biosynthetic enzymes unless they are strictly necessary under very limiting thiamine

390   concentrations. The existence of these regulatory hierarchies between transcriptional and

391   translational acting riboswitches are uncommon in model organisms studied so far. Expansion

392   of model systems to include a greater diversity of bacterial phyla may reveal new examples that

393   regulate responses to vitamins, amino acids, or other nutrients involved in core metabolic

394   pathways (24, 36, 27).

395   The study of how thiamine impacts microbial metabolism, global gene expression and

396   microbial physiology may help us understand nutrition-dependent mechanisms responsible for

397   modulating microbial community dynamics in the gastrointestinal tract. Our work demonstrates

398   that *B. vulgatus* is effectively blind to exogenous thiamine, suggesting that it will not compete for

399   available thiamine with the host or other gut microbes. Reliance on biosynthesis for production

400   of essential vitamins may be a strategy used by certain species to establish a foothold in

401   complex communities where availability of these molecules varies depending on diet and

402   competition. The lack of thiamine transport capacity of *B. vulgatus* was not obvious based on

403   existing genome sequence annotation of open reading frames, highlighting the importance of

404   functional genomics and accurate annotation of cis-acting regulatory elements like riboswitches.

405   Further characterization of mechanisms regulating thiamine acquisition and biosynthesis might

406   lead to development of new strategies to modulate microbial community composition and

407   metabolic function through targeted approaches controlling the activities of vitamin-dependent

408   riboswitches (10, 41). These strategies could have a variety of exciting therapeutic applications

409   that leverage the beneficial roles of microbial communities in human health and nutrition.

410

411   **Materials and Methods**

412   *Bacterial Strains and Plasmids, Culturing and Genetic Manipulation*

17

413   Bacterial strains and plasmids and oligonucleotides used for strain construction are

414   described in Table S2.

415   Culturing of *Bacteroides* strains *B. thetaiotaomicron* VPI-5482, *B. uniformis* ATCC 8492,

416   and *B. vulgatus* ATCC 8482 occurred anaerobically at 37°C in liquid tryptone yeast extract

417   glucose (TYG) medium (42) or a modified minimal medium with 0 to 10 µM (final concentration)

418   of thiamine hydrochloride as described previously (10, 43, 44) or Difco brain heart infusion (BHI)

419   agar with the addition of 10% defibrinated horse blood (QuadFive, Ryegate, MT, USA). Cultures

420   were grown in a vinyl anaerobic chamber with an input gas mix consisting of 70% nitrogen, 20%

421   carbon dioxide, and 10% hydrogen (Coy Laboratory Products, Grass Lake, MI, USA). *E. coli*

422   S17-1 λ *pir* strains used for cloning and conjugation of reporter vectors were grown in LB

423   medium at 37°C aerobically. Antibiotics were added to the media when appropriate at final

424   concentrations as follows: ampicillin 100 µg/mL, gentamicin 200 µg/mL, and erythromycin 25

425   µg/mL.

426   Nanoluciferase reporter vectors were constructed via traditional cloning of the

427   nanoluciferase gene (45, 46) into the vector pNBU2 (36, 47). DNA fragments encompassing

428   TPP riboswitches and the first 5 codons of the downstream gene were amplified from genomic

429   DNA by PCR using HiFi Taq MasterMix (KAPA Biosystems, Wilmington, MA, USA) and inserted

430   in frame preceding the nanoluciferase gene in the pNBU2 vector. The constructs were

431   transformed into conjugation donor *E. coli* S17-1 λ *pir*, and confirmed via PCR amplicon size

432   and sequencing. After confirmation, vectors were then conjugated into *B. thetaiotaomicron* VPI-

433   5482, *B. uniformis* ATCC 8492, and *B. vulgatus* ATCC 8482 making a single insertion into the

434   genome of each strain (36, 47). A complete list of primers and plasmids used in this study are

435   provided in Table 3.

436
437   *Expression analysis*

438   Replicate cultures of wild-type *B. uniformis* and *B. vulgatus* were grown overnight in 5

439   mL minimal medium supplemented with a final concentration of 10 μM thiamine HCl (THI, >

440   99% pure) (Sigma Aldrich, St. Louis, MO, USA). Aliquots of each culture were pelleted by

441   centrifugation (1 min at 13,300 x g), culture supernatants were decanted and the cells were

442   washed 4 times in minimal medium with 0 nM thiamine. Cells were subcultured at a dilution of

443   1:2000 into 10 mL of minimal medium with either 0 or 15 μM (final concentration) thiamine HCl

444   in two biological replicates. Cell growth was monitored and cells were harvested between OD_{600}

445   0.4 and 0.6 on a UV spectrophotometer. Total RNA was extracted using the Qiagen RNeasy kit

446   and stored at -80°C. Total RNA was DNAse treated with DNase I (Thermo Fisher, Waltham,

447   MA, USA) and NEB DNase treatment protocol. DNAse treated RNAs were re-purified using the

448   Qiagen RNeasy kit (Hilden, Germany), quantitated using a Qubit 2.0 (Life Technologies,

449   Carlsbad, CA, USA), and stored at -80°C. This was the same method used for a previously

450   published RNA-seq experiment for *B. thetaiotaomicron* (10). RNA was submitted for integrity

451   analysis, ribosomal RNA depletion, and library construction at the W.M. Keck Center for

452   Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign. RNA

453   sequencing was performed using an Illumina 2500 HiSeq generating 100 nucleotide paired end

454   reads for all samples.

455   For targeted gene expression analysis, total RNA was purified as described above and

456   used to generate cDNA libraries using the first strand cDNA synthesis kit (Thermo Fisher,

457   Waltham, MA, USA). RT-qPCR was performed on a Biorad CFX connect instrument (Biorad,

458   Hercules, CA, USA) and SYBR Fast MasterMix 2x Universal (Kapa Biosystems, Wilmington,

459   MA, USA) following the manufacturer's instructions for triplicate biological samples with three

460   technical replicates. Five probe sets were used in three strains to amplify products specific to:

461   16S ribosomal RNA, *thiC, thiS*, *OMthi*, and *pnuT.* 16S rRNA primers were used as the control

462   (36) and novel primers for *thiC, thiS*, *OMthi,* and *pnuT* for *B. thetaiotaomicron* (10), *B. uniformis*,

463   and *B. vulgatus* were designed using Primer3 (48). Standard curves were used to evaluate the

464   efficiency of the amplification, all 5 genes had $R^2 \geq 0.98$ and slope between -3.30 and -3.40.

465   Relative expression changes were calculated using the $\Delta\Delta C_q$ method (49).

466

467   *Nanoluciferase Reporter Fusion Assays*

468   Reporter assays were performed through a modified and combined protocol based on

469   Lim *et al.* 2017 and Mimee *et al.* 2015. *Bacteroides* strains with integrated pNBU2 TPP

470   riboswitch nanoluciferase reporters were grown in a modified minimal medium and then washed

471   and back diluted to an OD_{600} of 0.004 in 1.5 mL Axygen deep 96-well plates (Corning, Inc.,

472   Corning, NY) over a gradient of thiamine HCl (Sigma-Aldrich, St. Louis, MO). Growth was

473   monitored by via a BioTek Synergy HTX Multi-Mode microplate reader (BioTek, Winooski, VT)

474   (10). When cells reached mid-log phase growth (0.35-0.58 OD_{600}) growth was stopped. 1 mL of

475   mid log phase culture was spun down in the Axygen deep well plate at 3000 x g for 10 minutes

476   and medium was poured off the pellets. Cell pellets were then resuspended in 50 µL of 10X

477   BugBuster protein extraction reagent (MilliporeSigma, Darmstadt, Germany). Cell suspensions

478   were then incubated at room temperature with gentle shaking for 10 minutes. Nanoluciferase

479   extracts were mixed 1:1 (10 µL:10 µL) with Nano-Glo Luciferase mixture. Mixtures were then

480   incubated for 5 minutes at room temperature. Luminescence was read on a BioTek Synergy

481   HTX Multi-Mode microplate reader (BioTek, Winooski, VT). Relative Luminescence Units were

482   normalized to the OD_{600} of cell cultures. Comparisons were made across thiamine concentrations

483   using pairwise Student's *t* tests in GraphPad Prism v6, and *P* values of ≤0.05 were considered

484   significant.

485   In Silico *identification of TPP riboswitches in* Bacteroides *species*

486   Detection of TPP riboswitches among Bacteroidetes was done as described in (10).

487   Infernal 2.0 was used to identify riboswitches using the covariance model of TPP riboswitches in

488   RFAM (RF00059) (50, 51) in 114 Bacteroidetes genomes available in RefSeq from the Human

489   Microbiome Project (52, 53). To confirm that Infernal correctly predicted TPP riboswitches,

490   TIGRFAM, PFAM, and Hidden Markov Models with HMMR were used to identify thiamine

491   pyrophosphate biosynthesis and transport genes among the same 114 RefSeq Bacteroidetes

492   genomes (53-55).

493   To analyze the trend in distances between riboswitches and downstream genes,

494   nucleotide distances between coordinates identified with Infernal 2.0 and TIGRFAM, PFAM, and

495   HMMR were taken and subtracted from one another (51, 54-57). Annotations and genes were

496   then manually inspected utilizing Artemis to confirm that genes and riboswitches were called

497   correctly and that distances were accurate and agreed with previous predictions.

498

499   **ACCESSION NUMBER**

500   RNAseq data generated for this project have been submitted to the NCBI SRA database as

501   study SRP148918

502

516

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679   **Tables:**

Table 1. Reads of quality trimmed and mapped RNA sequencing

Species	Treatment	Replicate	SRA Accession	Raw Reads	Final Processed Reads	Mapped Reads	Percent Mapped
B. thetaiotaomicron	–Thi	1	SRX3130807*	9,170,438	8,231,596	7,712,374	93.7%
		2	SRX3130806*	8,258,740	7,454,637	6,830,124	91.6%
	+Thi	1	SRX3130804*	9,017,267	8,169,029	7,783,046	95.3%
		2	SRX3130805*	8,444,554	7,652,018	7,144,413	93.4%
B. uniformis	–Thi	1	SRX4123144	15,534,762	13,334,281	13,036,981	97.8%
		2	SRX4123145	15,576,078	13,358,338	12,771,513	95.6%
	+Thi	1	SRX4123142	17,382,438	14,900,079	14,437,339	96.9%
		2	SRX4123143	15,505,061	13,290,981	12,756,005	96.0%
B. vulgatus	–Thi	1	SRX4123140	19,391,595	13,334,281	13,036,981	97.8%
		2	SRX4123141	18,821,066	13,358,338	12,771,513	95.6%

+Thi	1	*SRX4123138*	18,447,248	14,901,073	14,437,549	96.9%
	2	*SRX4123139*	18,300,462	13,748,120	13,183,146	95.9%

680   ***denotes results previously published in Costliow and Degnan 2017**

Table 2. Average distance distributions of TPP riboswitches from the first gene of their regulon

Gene Function	Gene	TIGRFAM / PFAM	TPP Riboswitches	Riboswitch Distance (Avg. ± St. Dev.)
Biosynthesis	*thiS*	TIGR01683 / PF02597	77	64 ± 14
	thiD	TIGR00097 / PF08543	17	24 ± 34
	thi4	TIGR00292 / PF01946	16	55 ± 19
	thiE	TIGR00693 / PF02581	9	106 ± 82
	thiC	TIGR00190 / PF05690	3	132 ± 0
		Total:	122	60 ± 34
Transport	*OMthi*	N.A. / PF00593, PF07715, PF14905	85	23 ±23
	pnuT	PF04973 / TIGR01528	12	3 ± 0
		Total	97	21 ± 23

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683 **Figures:**

684
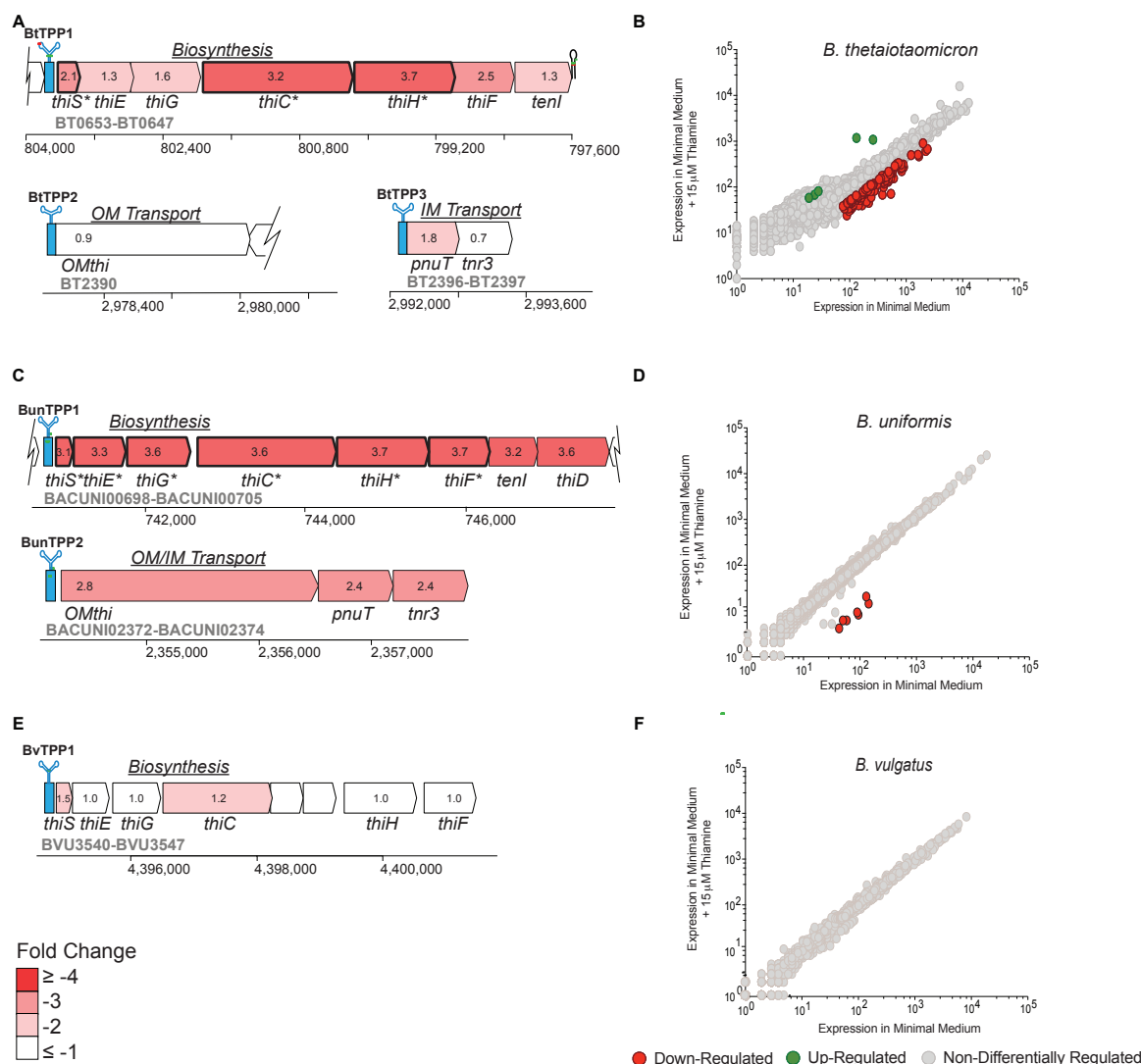
685   **FIG 1. Transcriptional response to exogenous thiamine**

686   Operons controlled by TPP riboswitches are shown for (A) *B. thetaiotaomicron,* (C) *B.*

687   *uniformis,* and (E) *B. vulgatus.* Expression changes across operons are visualized in a

688   schematic of each operon and color coded according to fold-change values comparing RNA-seq

689   reads for cells grown with and without added thiamine. Negative fold-change values indicate

690   that reads were reduced in cells grown in the presence of thiamine. Asterisks (*) and bold

691   numbers indicate significance expression differences (*q-value* $\leq$ 0.05 and a change in

692   expression $\geq$ 2-fold). All operons and distances are represented to horizontal scale. RNA-seq

693   data from (B) *B. thetaiotaomicron* (previously in Costliow and Degnan 2017)*,* (D) *B. uniformis,*

694   and (F) *B. vulgatus* grown in a defined minimal medium without thiamine (0 nM thiamine added)

695   and with thiamine (15 µM thiamine added)*.* Grey circles indicate genes that are not differentially

696   regulated. Green circles indicate genes that are significantly up regulated in the presence of

697   thiamine. Red circles indicate genes that are significantly down regulated in the presence of

698   exogenous thiamine. Data were analyzed using Rockhopper. The experiment was carried out in

699   biological duplicate.

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FIG 2. RT-qPCR confirmation of transcript level responses to exogenous thiamine

Transcript abundance of TPP riboswitch-regulated biosynthesis and transport genes was measured by RT-qPCR in (A) *B. thetaiotaomicron,* (B) *B. uniformis,* and (C) *B. vulgatus*. Primer sets were specific for genes indicated below bar graphs. Transcript levels of cells grown in the absence of thiamine (-) were normalized to levels in cells grown in the presence of 10,000 nM thiamine. Error bars represent the standard deviation (S.D.) for each experiment carried out in biological triplicate. Statistical significance for the differential abundance of gene transcripts were determined via a Student's t-test. Changes in expression were considered significant when the fold change was ≥ 2-fold with a *p*-value ≤ 0.05.
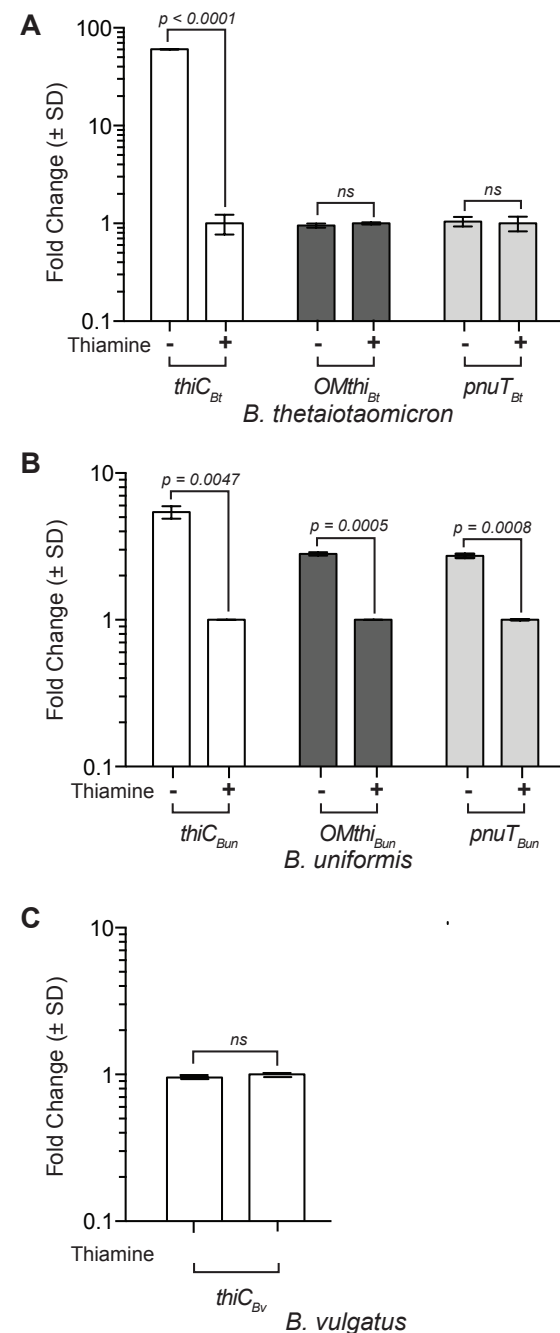

Fig. 2
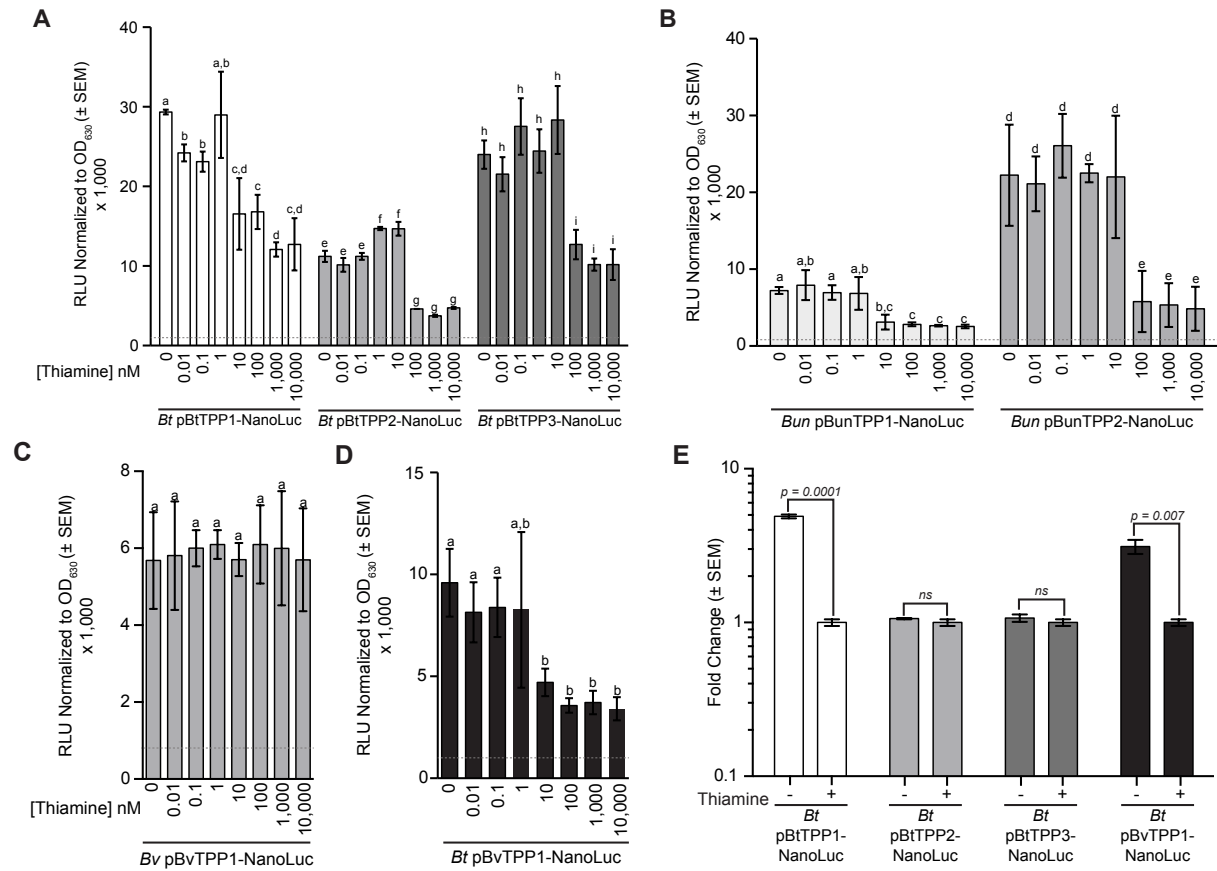
Fig. 3

726 **FIG 3. Nanoluciferase assays of *Bacteroides* TPP riboswitches**

727   Nanoluciferase translational fusions to genes downstream of all six TPP riboswitches

728 found in (A) *B. thetaiotaomicron,* (B) *B. uniformis,* and (C) *B. vulgatus* were tested. The *B.*

729 *vulgatus* reporter fusion was integrated into the *B. thetaiotaomicron* chromosome (D) to test the

730 mechanism of regulation in response to thiamine because *B. vulgatus* does not respond to

731 exogenous thiamine. RT-qPCR was carried out to measure reporter fusion mRNAs in *B.*

732 *thetaiotaomicron* with thiamine (+) or without thiamine (-) to confirm the mechanism of regulation

733 by predicted riboswitches from *B. thetaiotaomicron* and *B. vulgatus* (E). Relative light units

734 (RLU) were normalized to the OD_{630} of the bacterial cultures before cell lysis and activation of

735 the nanoluciferase reaction. Cells were harvested in mid-log phase for all samples (OD_{630}

736 between 0.4-0.6). The activity of each fusion was represented as the averaged RLU/ OD_{630} x

737 1000 from six independent biological replicates. Error bars represent the standard error of the

738 mean (SEM). Letters (a, b, c, etc.) indicate significant differences based on a *p-value* ≤ 0.05

739 using a Student's t-test. Grey dashed lines in A, B, C, and D indicate average RLU/ OD_{630} x

740 1000 from 3x biological replicates of a promoter-less nanoluciferase vector control for each

741 experiment.

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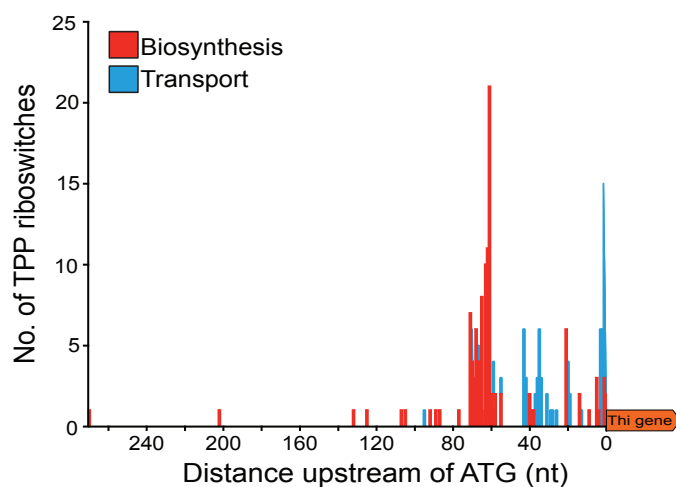
743   **Fig. 4**

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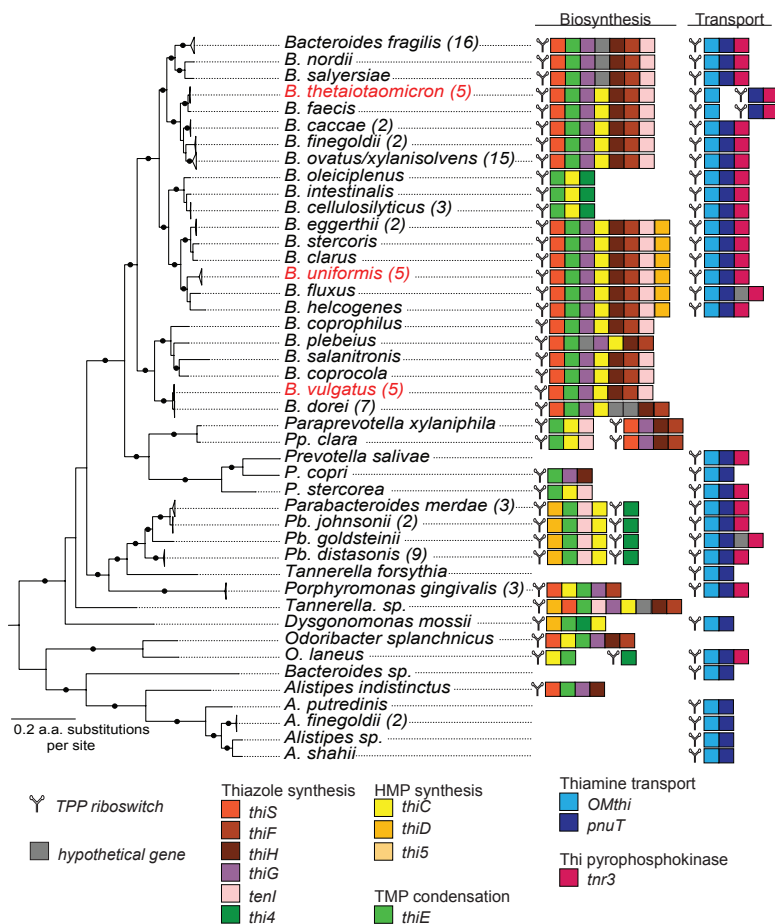
745   **FIG 4. TPP riboswitches throughout the Bacteroidetes**

746   Infernal and the RFAM 00059 covariance model were used to identify TPP riboswitches

747   across the Bacteroidetes. (A) ORFs downstream were inspected to determine the operons

748   directly preceded by putative TPP riboswitches. Function and syntenic context were assigned to

749   all predicted riboswitch regulated operons. (B) The distance of each TPP riboswitch from its

750   predicted operon was recorded and plotted and divided into the subcategories based on

751   functional class of genes regulated by the riboswitch - thiamine biosynthesis (red) or thiamine

752   transport (blue).

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