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Identification of a novel *N*-acetylmuramic acid (MurNAc) transporter in *Tannerella forsythia*

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Running title: MurNAc utilization in *Tannerella forsythia*

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ABSTRACT

Tannerella forsythia is a Gram-negative periodontal pathogen lacking the ability to undergo *de novo* synthesis of amino sugars *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) that form the disaccharide-repeating unit of the peptidoglycan backbone. *T. forsythia* relies on the uptake of these sugars from the environment, which is so far unexplored. Here, we identified a novel transporter system of *T. forsythia* involved in the uptake of MurNAc across the inner membrane and characterized a homolog of the *Escherichia. coli* MurQ etherase involved in the conversion of MurNAc-6P to GlcNAc-6P. The genes encoding these components were identified on a three gene cluster spanning Tanf_08375 to Tanf_08385 located downstream from a putative peptidoglycan recycling locus. We show that the three genes, Tanf_08375, Tanf_08380, and Tanf_08385, encoding a MurNAc transporter, a putative sugar kinase, and a MurQ etherase, respectively, are transcriptionally linked. Complementation of the Tanf_08375 and Tanf_08380 genes together *in trans*, but not individually rescued the inability of an *E. coli* mutant deficient in the PTS (phosphotransferase system)-dependent MurNAc transporter MurP as well as that of a double mutant deficient in MurP and components of the PTS system to grow on MurNAc. In addition, complementation with this two-gene construct in *E. coli* caused depletion of MurNAc in the medium, further confirming this observation. Our results show that the products of Tanf_08375 and Tanf_08380 constitute a novel non-PTS MurNAc transporter system that seems to be widespread among bacteria of the *Bacteroidetes* phylum. To the best of our knowledge, this is the first identification of a PTS-independent MurNAc transporter in bacteria.

IMPORTANCE

In this study we report the identification of a novel transporter for peptidoglycan amino-sugar *N*-acetylmuramic acid (MurNAc) in the periodontal pathogen *T. forsythia*. It has been known since the late 1980s that *T. forsythia* is a MurNAc auxotroph relying on environmental sources for this essential sugar. Most sugar transporters, and the MurNAc transporter MurP in particular require a PTS phosho-relay to drive the uptake and concurrent phosphorylation of the sugar through the inner membrane in Gram-negative bacteria. Our study uncovered a novel type of PTS-independent MurNAc transporter, and although so far unique to *T. forsythia*, may be present in a range of bacteria both of the oral cavity and gut especially of the phylum Bacteroidetes.

INTRODUCTION

Tannerella forsythia is a Gram-negative, obligate anaerobe strongly associated with periodontitis, which affects the soft and hard tissues supporting the teeth ultimately leading to tooth loss (1, 2). This bacterium is frequently found with the oral bacterial pathogens *Treponema denticola* and *Porphyromonas gingivalis*, together forming a pathogenic consortium termed the “red complex” (3), which in turn is part of a much wider dysbiotic microbiota that is thought to cause this widespread inflammatory disease (4). Strikingly, unlike other bacteria, *T. forsythia* depends on exogenous *N*-acetylmuramic acid (MurNAc) for growth (5). It was observed 27 years ago by Wyss that the cultivation of *T. forsythia* required spent broth from *Fusobacterium nucleatum* (5), or the presence of free MurNAc (6, 7) in the medium. Since MurNAc together with *N*-acetylglucosamine (GlcNAc) forms the peptidoglycan amino sugar backbone in all

bacteria this indicated *that T. forsythia* is unable to synthesize its own peptidoglycan amino sugars. The reasons for this auxotrophy for the amino sugar MurNAc became evident after the close inspection of the *T. forsythia* genome sequence which became available in 2005 (8, 9). It was noted that the MurA and MurB enzyme homologs required for the *de novo* synthesis of MurNAc and GlcNAc are not present in the bacterium (10). In addition, the bacterium lacks GlmS, GlmM, and GlmU enzymes for biosynthesis of GlcNAc. Furthermore, evidence collected by analyzing genomes of *T. forsythia* strains deposited at the Human Oral Microbial Database indicated that this bacterial species lacks a canonical phosphotransferase (PTS) type MurNAc transporter (MurP), which in *E. coli* and related Gram-negative bacteria is required for MurNAc uptake and concomitant phosphorylation (11). PTS-type sugar transporters generally mediate the uptake and phosphorylation of sugars; a prototypical PTS system consists of an enzyme I EI, a histidine protein HPr, the sugar-specific components EIIA and EIIB, and a transmembrane sugar-specific transporter protein EIIC (12). The lack of PTS systems in *T. forsythia* suggests that this bacterium utilizes an alternative transport system to utilize exogenous MurNAc from the environment.

Our *in silico* investigation of the *T. forsythia* genome revealed genes coding for putative peptidoglycan degradation and recycling functions (10), among these was a homolog (Tanf_08385; accession no. WP_046825532) of the *E. coli* MurQ (13) etherase and two adjacent genes encoding a putative integral membrane protein (Tanf_08375; WP_046825530.1) and a putative sugar kinase (Tanf_08380; WP_046825531.1). Here we report the preliminary characterization of a novel PTS-independent transport system for MurNAc uptake comprising Tanf_08375 and

89 Tanf_08380 proteins in *T. forsythia*, which we propose be named TfMurT and TfMurK
90 respectively, and *T. forsythia* MurQ etherase (TfMurQ) involved in the metabolic
91 conversion of MurNAc-6P to GlcNAc-6-P.

92

93 **MATERIALS AND METHODS**

94 **Bacterial strains and growth conditions**

95 The *T. forsythia* ATCC 43037 wild-type and mutant strains used in this study
96 were grown anaerobically in BF broth, or on agar plates as described previously (14).
97 *Escherichia coli* strains were grown in Luria-Bertani broth (LB) aerobically at 37°C. *E.*
98 *coli* strains were also grown in minimal M9 media (15) supplemented with either 0.2%
99 glucose, 0.2% glycerol, or 0.025% MurNAc, where needed. *E. coli* Δ*murQ* and Δ*murP*
100 mutants were from the Keio collection at the Yale Coli Genetic Stock Center
101 (http://cgsc.biology.yale.edu). All strains and plasmids used in this study are
102 summarized in Table S1 (supplementary material).

103

104 **Molecular biology techniques**

105 Standard molecular cloning techniques were performed according to (16). All
106 cloning experiments were performed using the electrocompetent *recA* mutant cloning
107 strain *E. coli* Stellar (Clontech laboratories, CA, USA).

108

109 **Reverse transcription-polymerase chain reaction**

Total RNA was isolated from bacteria using the RNeasy kit (Qiagen). Single-stranded cDNA was synthesized using reverse transcriptase (Invitrogen Superscript III) and random hexamer primers as per the manufacturer's protocol. The synthesized cDNA was amplified by PCR with primer sets spanning target genes *murQ*, *murT*, and *murK* (Fig. 5b): region 'a' with TF1067F/TF1068R; region 'b' with TF1068F/TF1069R; region 'c' with TF1067F/TF1069R. Primer sequences are listed in Table S2 (supplementary material).

Production of recombinant TfMurQ protein

Recombinant plasmid pET-TfMurQ was constructed by cloning a TfMurQ ORF fragment in-frame with a C-terminal 6xHis-tag of the pET30a expression vector (Novagen). Briefly, a PCR fragment amplified with primers TF1069-F and TF1069-R (Table S2) from *T. forsythia* ATCC 43037 genomic DNA was digested with NdeI and XhoI and cloned via NdeI/XhoI sites into pET30a to generate pET-TfMurQ. Subsequently, *E. coli* BL21/DE3 strain carrying the pET-TfMurQ plasmid was grown in LB medium with kanamycin (50 µg/ml) at 30°C to an OD_{600} of 0.3. Protein expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration of 1 mM) for additional 3 h at 30°C. Bacteria were collected by centrifugation at 7,000 X g for 10 min, washed with PBS twice, and lysed by sonication for 30 s. Lysates were centrifuged at 10,000 X g for 20 min and supernatants were collected. Supernatants were loaded onto a column containing 500 µl of HIS-Bind resin (Qiagen) and the column was equilibrated with 10 ml of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). Bound recombinant protein was eluted with 1 ml of elution buffer

(50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) and dialyzed extensively against phosphate–buffered saline, pH 7.2 at 4^0C. The dialyzed protein fraction was analyzed by SDS-PAGE on 12% gels stained with Coomassie Brilliant Blue R250.

Detection of etherase catalyzed reaction intermediate

Etherase activity was assessed by utilizing MurNAc-6P as substrate in a Morgan Elson reaction (17). This etherase catalyzed reaction generates a chromogenic intermediate that can be detected by reacting with Ehrlich's reagent dimethylaminobenzaldehyde, to yield a purple product. To detect the formation of this chromogen compound in an enzyme catalyzed reaction, an Ehrlich-Morgan-Elson assay was performed (18). Briefly, 2 µl of purified rTfMurQ enzyme (1, 2 or 4 µg protein) was added to 20 µl of MurNAc-6P (10 mM in water and the reaction mixture was incubated for 60 min at 45°C. After addition of 100 µl of Ehrlich's reagent, incubation was continued for 20 min at 37°C.

Radioactive etherase assay

The ^{32}P-radiolabled substrates MurNAc-6P and GlcNAc-6P, respectively, were prepared according to a published protocol (19) with minor modifications. Aqueous solutions of 50 mM MurNAc or GlcNAc, respectively, were added to a reaction mixture containing 100 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$ 100 mM ATP, 140 kBq of [γ-^{32}P] ATP and 20 µg recombinant *Clostridium acetobutyticum* MurK protein in a total volume of 100 µl and the reaction was incubated overnight at 37°. To start the etherase assay,

155 a reaction mixture containing 15 µl of MurNAc-6-P, 0,4 µg of rTfMurQ and 100 mM Tris-
156 HCl, pH 7.6 in a total volume of 50 µl was incubated at 37°. 2 µl of this mixture were
157 spotted immediately and after 15 and 30 min of incubation on a TLC plate (Silica 60
158 F254, Merck, Darmstadt, Germany). Reaction products were separated in a basic
159 solvent with *n*-butyl alcohol/ methanol/ 25% (w/v) ammonium hydroxide/ water (5:4:2:1).
160 The radioactive products were detected using a Typhoon Trio Biomolecular imager (GE
161 Healthcare).

162

163 **Construction of expression vectors and complementation of *E. coli* Δ*murP***
164 **mutants and Δ*murQ***

165 DNA fragments coding for *T. forsythia* MurT-MurK, MurT, MurK, and MurQ ORFs
166 were amplified with primer sets listed in Table S2 from *T. forsythia* ATCC 43037
167 genomic DNA, digested with Nde1 and HindIII, and cloned into pTrc99 at NdeI/HindIII
168 restriction sites to generate the plasmids pTr-MurTK, pTr-MurT, pTr-MurK, and TfMurQ,
169 respectively. Plasmids were confirmed by sequencing. For complementation, *E. coli*
170 mutants were transformed with the plasmids above via electroporation (16).

171

172 **MurNAc depletion assay**

173 B
174 acterial cells from overnight cultures of *E. coli murP* mutant strain harboring either
175 pTrc99, pCS19YfeV, or pTr-MurTK were washed and diluted in M9 minimal medium to
176 an OD_{600} of 0.05. Before the start of an assay, 10 ml of cell suspension from each

177 strain in triplicate was supplemented with glucose and MurNAc to final concentrations of
178 5.5 mM and 3.5 µM, respectively, as carbon sources. The cell suspensions were
179 incubated with shaking at 37^0C. At regular time intervals 0.6 ml aliquots were withdrawn,
180 OD_{600} determined and cell free supernatants were recovered by centrifugation and
181 saved. MurNAc concentration in the cell free supernatants was then assayed according
182 to a previously described colorimetric assay specific for *N*-acetyl amino sugars (17).
183 Briefly, 0.1 ml of potassium tetraborate solution was added to 0.5 ml of sample (culture
184 supernatant) followed by boiling for 3 min and cooling to room temperature.
185 Subsequently, 3 ml of *p*-dimethylaminobenzaldehyde (DMAB) reagent (Sigma) was
186 added and the tubes were placed in a water bath at 37°C for 20 min. Color developed
187 was read at 585 nm and the amount of MurNAc was calculated from a standard curve of
188 MurNAc in the range of 0.625 - 5 µM.

189

190 **RESULTS**

191 ***T. forsythia* contains a putative MurNAc utilization locus**

192 *In silico* analysis of the *T. forsythia* ATCC 43037 draft genome
193 (JUET00000000.1; http://www.ncbi.nlm.nih.gov/nuccore/JUET00000000.1/) identified a
194 three-gene locus (Tanf_08375-Tanf_08385) in the contig_82 DNA sequence
195 (NZ_JUET01000082） that included genes coding for an inner membrane protein
196 (Tanf_08375; WP_046825530.1), a putative sugar kinase (Tanf_08380;
197 WP_046825531.1) and a putative MurQ-type etherase (Tanf_08385; WP_046825532),.
198 This region is 97% identical to a DNA region of *T. forsythia* strain 92A2 spanning
199 BFO_0041 to BFO_0044 (NC_016610;

200 http://www.ncbi.nlm.nih.gov/nuccore/NC_016610.1). Interestingly, the Tanf_08375-
201 Tanf_08385 gene cluster is located immediately downstream of a putative
202 peptidoglycan recycling operon including a muropeptide permease AmpG homolog
203 (Tanf_08365) (20) (Fig. 1a). The product of the putative etherase gene (TfMurQ) shows
204 46% and 84%, identity with the *N*-acetylmuramic acid-phosphate (MurNAc-P) etherase
205 MurQ of *E. coli* (gi:16130353) and predicted MurQ from *Bacteroides fragilis*
206 (gi:763470620), respectively. The putative *T. forsythia* MurQ contains a SIS domain
207 (sugar isomerase domain, accession no. cd04795) characteristic of phosphosugar
208 isomerases and phosphosugar binding proteins.

209

210 **MurT-MurK function as a PTS-independent MurNAc transporter**

211 In *E. coli* and many other bacteria, free MurNAc is transported across the inner
212 membrane and is simultaneously phosphorylated by the PTS dependent MurP
213 permease, which is the MurNAc-specific IIBC domain of the PTS system (11). Further
214 processing of phosphorylated MurNAc through the action of MurQ leads metabolic
215 products to either enter a glycolytic pathway for generating energy, or biosynthetic
216 pathway for generating peptidoglycan amino sugar GlcNAc (18, 21) (Fig. 1b). As
217 mentioned above, *T. forsythia* lacks a canonical PTS-type transporter complex and,
218 thus utilizes alternative mechanism to transport and phosphorylate MurNAc. *In silico*
219 analysis indicated that TfMurT is a membrane protein with ten putative membrane
220 spanning helices (Fig. S1, supplementary material) while TfMurK is a putative sugar
221 kinase with a predicted nucleotide binding domain commonly found in sugar kinases
222 and heat shock proteins (NBD_sugar-kinase_HSP superfamily; accession no. cl17037).

223 Taken together, we predicted that in *T. forsythia* MurT functions as a MurNAc
224 transporter and MurK functions as a MurNAc kinase (Fig. 1b).

225 To determine the functional role of TfMurT and TfMurK in MurNAc transport, we
226 tested if providing the *T. forsythia murT* and *murK* genes *in trans* to an *E. coli* Δ*murP*
227 mutant, would rescue the inability of the Δ*murP* mutant to utilize MurNAc as a sole
228 carbon and energy source. The results showed that while the *E. coli* Δ*murP* mutant
229 (CM103) complemented with a plasmid (pTr-MurTK) co-expressing TfMurT and TfMurK
230 proteins grew on minimal agar supplemented with 0.025% w/v MurNAc (Fig. 2a, middle
231 row) or broth (Fig. 2b), neither the mutant alone nor the mutant complemented with the
232 empty plasmid pTcr99a grew on MurNAc. All strains grew on minimal agar with glucose
233 used as a control. Additionally, as a positive control, complementation with native *E.*
234 *coli murP in trans* via pCS19YfeV restored the growth defect of the *E. c*oli Δ*murP* strain
235 on MurNAc (Fig. 2a, middle row). Importantly, the growth of *E. coli* Δ*murP* in the
236 presence of MurNAc was rescued with the combined expression of *T. forsythia*
237 MurT/MurK, and was similar to the growth in the presence of native *E. coli* MurP. To
238 investigate whether MurNAc transport requires TfMurT/TfMurK co-expression,
239 complementation with either TfMurT or TfMurK in *E. coli* Δ*murP* was performed. The
240 results showed that neither TfMurT nor TfMurK alone could confer to the mutant the
241 ability to grow on MurNAc (Fig. 2a).

242 Next, since TfMurT and TfMurK proteins do not possess PTS-type signatures, we
243 wanted to confirm that the TfMurT membrane protein and the TfMurK kinase function
244 independently of a canonical PTS system. For this purpose, we provided the *T.*
245 *forsythia murT-murK* genes *in trans* to an *E. coli* double mutant (CM133) with deletion of

246 the *murP* (*yfeV*) gene and the entire *pts* operon (*ptsHIcrr*) coding for the components of
247 the PTS system. CM133 was generated by P1 transduction to transfer Δ*ptsHIcrr*::kan
248 mutation from JM-G77 to CM103. The results showed that complementation of CM133
249 with *murT-murK* restored the growth the mutant on MurNAc. As shown, CM133 grew
250 on MurNAc containing agar (Fig. 2a) or broth (Fig. 2b) when complemented with the
251 plasmid pTr-MurTK co-expressing TfMurT and TfMurK but did not grow on MurNAc
252 when complemented with the plasmid pTr-MurT or pTr-MurK expressing either protein
253 alone. As controls, complementation with native *murP* (pCS19yfeV) or empty vector did
254 not rescue the growth of CM133 on MurNAc; growth was rescued only when glycerol
255 (0.2%) was provided as the sole carbon source (N.B. this strain is unable to grow on
256 glucose given its general PTS defect). The parent strain MC4100, from which CM103
257 and CM133 were derived, carrying either plasmid grew on glycerol as well as MurNAc
258 (Fig. 2b). Together these data demonstrated that the products of TfMurT and TfMurK
259 function independently of a PTS system for transport and utilization of MurNAc.

260 To confirm that this putative transport complex was indeed involved in MurNAc
261 utilization, an experiment was designed where depletion of MurNAc by *E. coli* strains
262 was assessed in a minimal medium with glucose or MurNAc as a carbon source. Under
263 these conditions *E. coli ΔmurP* mutant carrying either an empty plasmid or plasmid
264 expressing the *E. coli* MurP (pCS19YfeV) or the *T. forsythia* MurTK (pTr-MurTK) grew
265 as expected and MurNAc depletion in the medium was not observed for *E. coli* cells
266 bearing empty plasmid. However, significant depletion of MurNAc was observed in the
267 case of *E. coli* Δ*murP* mutant complemented with pTr-MurTK expressing
268 TfMurT/TfMurK or pCS19YfeV expressing native *E. coli* MurP transporter (Fig. 3).

Taken together, these data demonstrate that TfMurK and TfMurT act in concert and TfMurT is a unique transporter for the utilization of exogenous MurNAc in *T. forsythia*.

Tanf_08385 encodes *T. forsythia* MurQ etherase and is co-transcribed with *murTK*

Since the MurQ etherase is important in the utilization of MurNAc in bacteria (Fig. 1b), we confirmed the activity of Tanf_08385 as a functional MurNAc-6-P etherase (TfMurQ). For this purpose, TfMurQ expressed as a His_6-tagged recombinant protein (rTfMurQ) in *E. coli* was purified to homogeneity by nickel affinity chromatography (Fig. S2, supplementary material), and confirmed the etherase activity using the Elson-Morgan enzymatic assay and conversion of MurNAc-6-P to GlcNAc-6-P by a radioactive assay using ^{32}P-labled MurNAc-6-P (18). The Elson-Morgan assay showed that the purified rTfMurQ had etherase activity since a color change was seen with Ehrlich's reagent when rTfMurQ was incubated with MurNAc-6-P (Fig. S3, supplementary material). Furthermore, rTfMurQ protein catalyzed the formation of a radioactive GlcNAc-6-P product when incubated with MurNAc-6-P in a TLC-based assay employing ^{32}P-labelled MurNAc (Fig. 4a). In addition, the functionality of TfMurQ was tested by *trans* complementation in an *E. coli* Δ*murQ* mutant. For this purpose, the *E. coli* Δ*murQ* mutant JW2421-1 was transformed with either an IPTG-inducible plasmid harboring the *Tf murQ* gene (pTr-MurQ), or an empty plasmid vector (pTrc99) and plated on minimal agar plates with glucose or MurNAc as the sole carbon source. Growth of the *E. coli* Δ*murQ* mutant JW2421-1 complemented with pTr-MurQ was rescued on minimal agar plates containing MurNAc (Fig. 4b). The *E. coli* Δ*murQ* mutant complemented with empty pTrc99 did not grow on MurNAc containing plates, but grew on media

supplemented with glucose. In contrast, the *E. coli* parent strain BW25113 harboring pTrc99 grew on minimal media containing glucose and MurNAc as sole carbon source (Fig. 4b). These data suggested that Tanf_08385 is the *T. forsythia* MurQ etherase (TfMurQ) involved in the metabolic conversion of MurANc-6-P to GlcNAc-6-P. Next, we wanted to determine whether *mur* genes are co-transcribed. For this purpose, RNA from *T. forsythia* ATCC 43037 cells was extracted and co-transcription of the *mur* genes was analyzed using RT-PCR as outlined in Fig 5. The data demonstrated that the *T. forsythia murT*, *murK,* and *murQ* genes were transcribed as a single transcript (Fig. 5b), since PCR products of the expected size were obtained with primer pairs (Table S2, supplementary material) designed to bridge the ends between the open-reading frames (ORFs) of adjacent genes, and, thus, yielding amplification products only when co-transcription was occurring. Taken together, our data showed that the *murT, murK, and murQ genes form an operon (murTKQ)* involved in MurNAc utilization.

DISCUSSION

T. forsythia, a common pathogen present in dental biofilms, is implicated in periodontitis. Its role in the disease process has been confirmed in animal models (22) and it has been demonstrated that the bacterium's ability to induce disease is enhanced when co-infected with other bacteria such as *Fusobacterium nucleatum* (23). Strikingly, *T. forsythia* depends on exogenous MurNAc, an essential peptidoglycan amino sugar, for growth. Its inability to *de novo* synthesize the peptidoglycan amino sugars MurNAc and GlcNAc was first described by Wyss (5), who noted that growth of *T. forsythia* could be rescued when spent media from cultures of *F. nucleatum* or free MurNAc was

315 supplied exogenously. Since, MurNAc is not known to be synthesized by the human
316 host, scavenging on peptidoglycan byproducts (muropeptides, anhydro-MurNAc)
317 released by cohabiting oral bacteria during their cell wall recycling is a plausible
318 mechanism by which *T. forsythia* obtains MurNAc *in vivo*. Therefore, growth and, thus,
319 the virulence potential of *T. forsythia* depend on its ability to obtain and utilize MurNAc,
320 or MurNAc-containing peptidoglycan fragments from the environment. To our
321 knowledge, no other bacterium has such a strict requirement for MurNAc. Moreover,
322 despite its clear ability to utilize exogenously supplied MurNAc, the *T. forsythia* genome
323 lacks homologs of PTS-type MurNAc transporters present in bacteria (12). In *E. coli*
324 and the majority of bacteria, the MurP PTS system is responsible for phosphorylation
325 and import of MurNAc (11, 21), and further utilization of MurNAc transported as
326 MurNAc-6P proceeds through the action of MurQ etherase (18). MurP contains both the
327 PTS domains EIIB and EIIC and requires Enzyme I, histidine protein HPr and the
328 phosphoryl transfer protein EIIA ($EIIA^{Glc}$) for function. We searched the *T. forsythia*
329 ATCC 43037 genome for a similar PTS-type MurNAc transport system, but our search
330 identified no MurP or any of the PTS homologs in the genome of *T. forsythia*. However,
331 we identified a genetic cluster (Tanf_08375-Tanf_08385) in the genome that contained
332 ORFs for a membrane protein (TfMurT), a sugar kinase (TfMurK), and an etherase
333 (TfMurQ). This genetic cluster is located immediately downstream from a locus likely to
334 be involved in peptidoglycan recycling as suggested by the presence of an ORF for a
335 putative peptidoglycan permease AmpG in the locus (Fig. 1a). Since TfMurT and
336 TfMurK ORFs were present in close association with an ORF for a MurQ-like etherase
337 (TfMurQ), we hypothesized that MurT and TfMurK might be involved in MurNAc

transport and utilization functions. During peptidoglycan recycling in bacteria, MurNAc is released as anhMurNAc (1,6-anhydro-MurNAc) and is phosphorylated to MurNAc-6P by the kinase AnmK (13). MurNAc-6-P is converted by the MurQ etherase into GlcNAc-6-P and both these sugars are reused for synthesis of new peptidoglycan or enter the general carbohydrate metabolism (13).

In this study, we showed that expression of TfMurT and TfMurK bipartite pair in an *E. coli* Δ*murP* mutant restored bacterial growth in minimal media supplemented with MurNAc. In addition, TfMurQ *trans* complementation in an *E. coli ΔmurQ* mutant restored the ability to utilize MurNAc and the purified recombinant TfMurQ protein converted MurNAc-6-P to GlcNAc-6-P in vitro. These data show that TfMurT and TfMurK, coding for an integral membrane transporter and a putative MurNAc sugar kinase, respectively, constitute a unique PTS-independent system for MurNAc transport and phosphorylation. Furthermore, TfMurQ is involved in the metabolic conversion of MurNAc-6-P to GlcNAc-6-P. The functionality of TfMurT and TfMurK was confirmed via *trans* complementation in *E. coli* host. Deletion of these ORFs in *T. forsythia* was potentially lethal as no mutants were recovered. While we predict that TfMurT and TfMurK proteins are likely present in close association or direct physical interactions as a bipartite pair (Fig. 1b) to carry out the function of transport and phosphorylation of MurNAc, we have no experimental evidence to support this notion and the presence of TfMurK as a cytoplasmic protein cannot be ruled out. Future studies will be needed to biochemically characterize the structure-function relationship of MurT/MurK proteins. Our preliminary attempts to obtain a soluble active form of 6xHis-tagged recombinant TfMurK protein have been unsuccessful as the recombinant protein expresses in an

insoluble, inactive form, even after attempted refolding from insoluble material. Alternative expression approaches are underway to obtain the protein in the soluble form. TfMurT/TfMurK proteins do not possess PTS-type signatures and together represent a novel transport system for MurNAc in *T. forsythia*. PTS-independent sugar transporters, not as common as PTS-dependent systems, have been previously reporter in bacteria. However, such systems have not been characterized at the molecular level. For instance, in streptococci (24, 25) and corynebacteria (26), there is evidence of PTS-independent glucose uptake. We predict that this mode of sugar uptake and utilization might be prevalent at least in the *Bacteroidetes* phylum of bacteria, since homologs of the *murT* and *murK* genes of *T. forsythia* are present in the genomes of a range of several gut *Bacteroides spp*. and oral *Prevotella spp*. *(*Fig. S4, supplementary material). Strikingly, *T. forsythia* and *Prevotella spp.* seem to have a minimal gene set as the others have extra genes in the cluster including kinases, ferredoxin, and a β-lactamase, which may reflect their unique niches. Thus, the TfMurTK system is the first evidence of a PTS-independent MurNAc transporter system to date and although so far unique to *T. forsythia*, may be present in a range of Gram-negative bacteria both of the oral cavity and gut.

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453 **FIGURE LEGENDS**

454 **Fig. 1.** *T. forsythia* MurNAc utilization locus and MurNAc utilization pathway. (A) Genetic
455 organization of the MurNAc utilization locus Tanf_08370-Tanf_08385 (black) of *T.*
456 *forsythia*. The genes associated with the locus encode a membrane protein TfMurT, a
457 sugar kinase TfMurK, and an etherase TfMurQ. The locus Tanf_08345-Tanf_08365
458 (light gray) present immediately upstream is potentially involved in peptidoglycan
459 recycling; Gtf - predicted glycosyltransferase; LytB - predicted amidase enhancer;
460 AmpG - predicted muropeptide transporter; YbbC- hypothetical protein, (B) Schematic
461 model of a MurNAc transport and utilization pathway in *E. coli* and *T. forsythia.* PEP,
462 phosphoenolpyruvate; E1, enzyme E1; HPR, Histidine protein; EIIA; enzyme IIA.

463

464 **Figure 2.** Growth of *E. coli* strains MC4100 (parental strain), CM103 *(ΔmurP*), and
465 CM133 *(ΔmurP, Δpts*) complemented with respective plasmids in M9 minimal agar and
466 liquid medium with 0.2% glycerol, or 0.025% MurNAc. (A) Plate legend and growth of *E.*
467 *coli* strains on agar. (B) Growth of *E. coli* strains in medium with MurNAc (Mu) or
468 glycerol (Gl) measured at OD_{600}. Results of one out of three independent cultivations
469 with similar outcome are given.

470

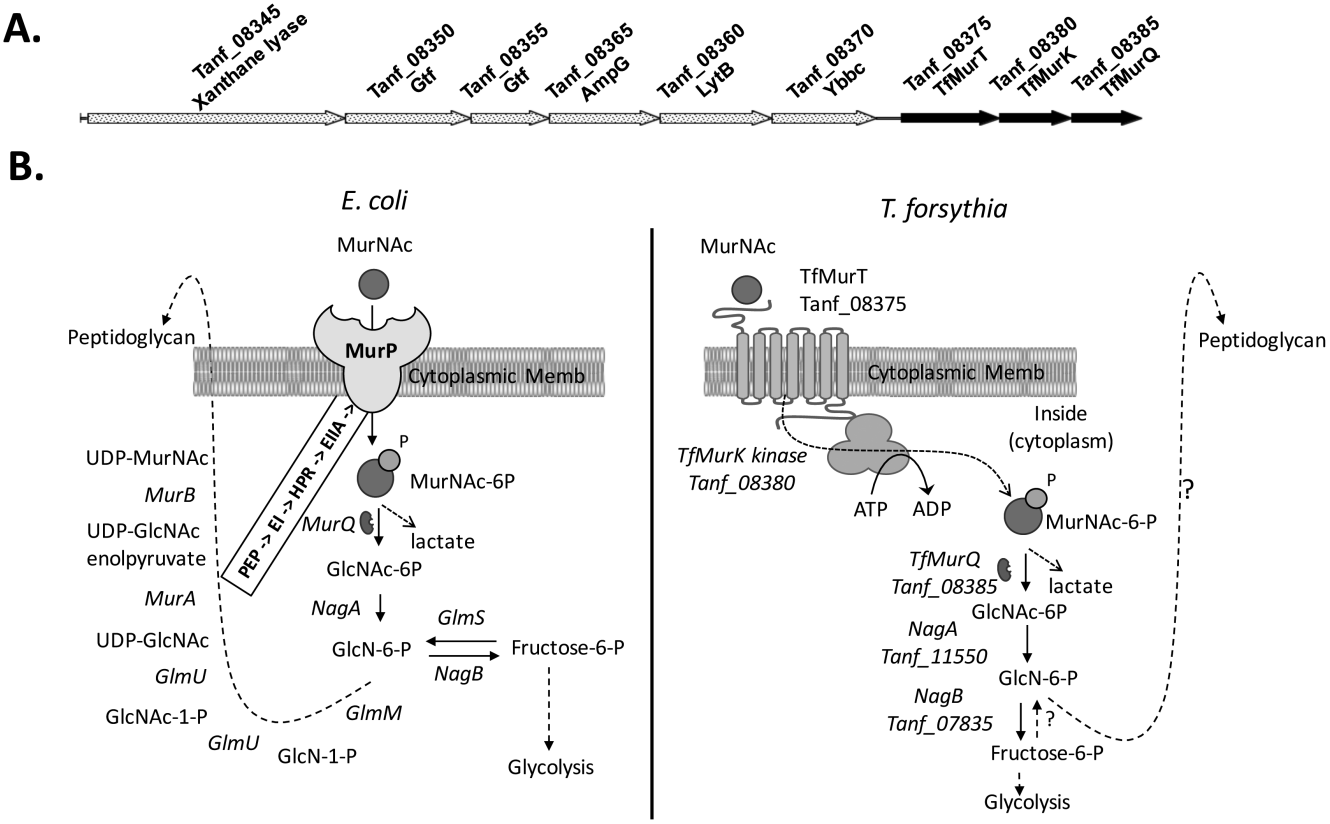
471 **Figure 3.** MurNAc depletion in minimal media incubated with *E. coli* Δ*murP* mutant
472 complemented with respective plasmids. *E. coli* strains were incubated in minimal
473 media supplemented with glucose and MurNAc and every 2 h post incubation spent
474 medium for each strain was assayed for MurNAc using a chromogenic assay specific
475 for *N*-Acetyl-aminosugars.

476

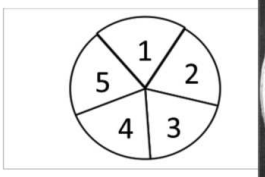
477 **Figure 4.** *T. forsythia* MurQ (TfMurQ) is a MurNAc-6-P etherase. (A) TLC analysis of
478 MurNAc-6^{32}P-phosphate conversion by TfMurQ. MurNAc was radioactively
479 phosphorylated at position C-6 by using recombinant *Clostridium acetobutyticum* MurK
480 and γ-^{32}P-ATP. MurNAc-6^{32}P-phosphate was then incubated with purified rTfMurQ
481 etherase and MurNAc-6^{32}P-phosphate to GlcNAc-6^{32}P-phosphate conversion was
482 monitored. Samples from different time points (lanes 1, 2 and 3) were spotted on a TLC
483 plate together with the standards MurNAc-6P (lane 4) and GlcNAc-6P (lane 5). The
484 radioactive products were detected using a phosphoimager. (B) Complementation of an
485 *E. coli* Δ*murQ* mutant (JW2421-1) with TfMurQ. The *E. coli* Δ*murQ* mutant, empty
486 vector control (pTrc99), and complemented strain were plated on minimal agar with
487 MurNAc (0.02% w/v) or glucose (0.2% w/v) as a control.

488

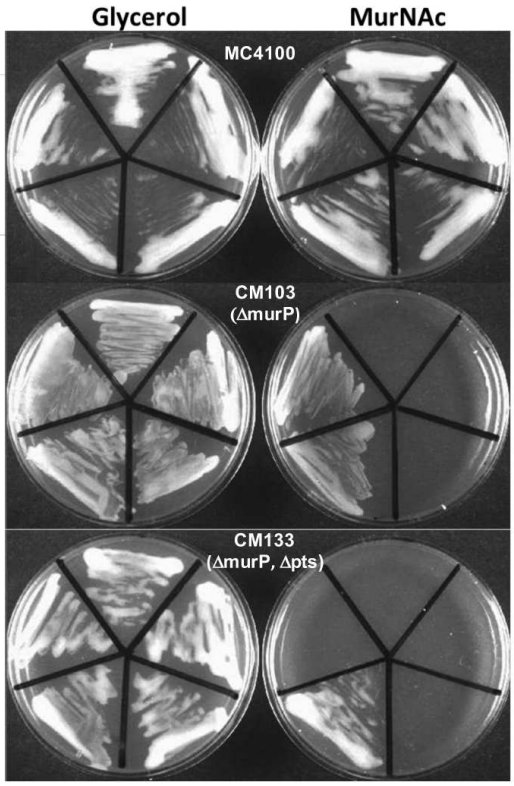
489 **Figure 5.** RT-PCR analysis with (A) primer sets spanning adjacent genes (fragments a,
490 b, or c). (B) PCR products were separated on a 1% agarose gel. No reverse
491 transcription (RNA only) controls were run in lanes 1, genomic DNA as template in
492 lanes 2, and cDNA as template for each primer set in lanes 3. MW; DNA ladder.



A.



1) pTrc99
2) pTr-MurT
3) pTr-MurK
4) pTr-MurTK
5) pCS19YfeV



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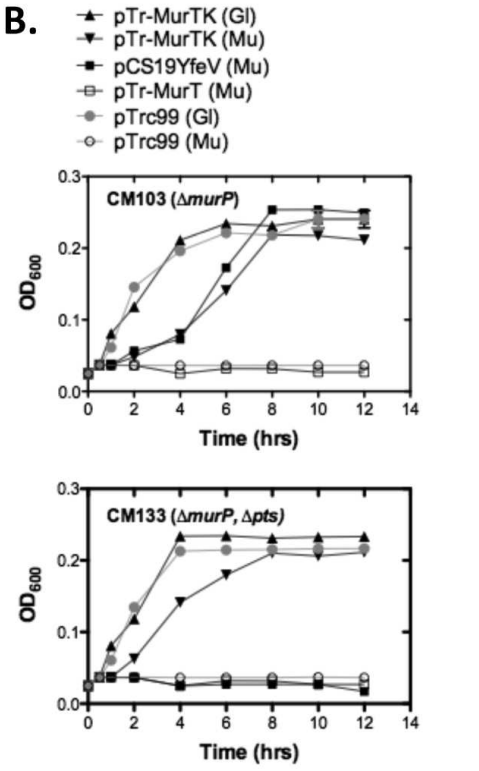
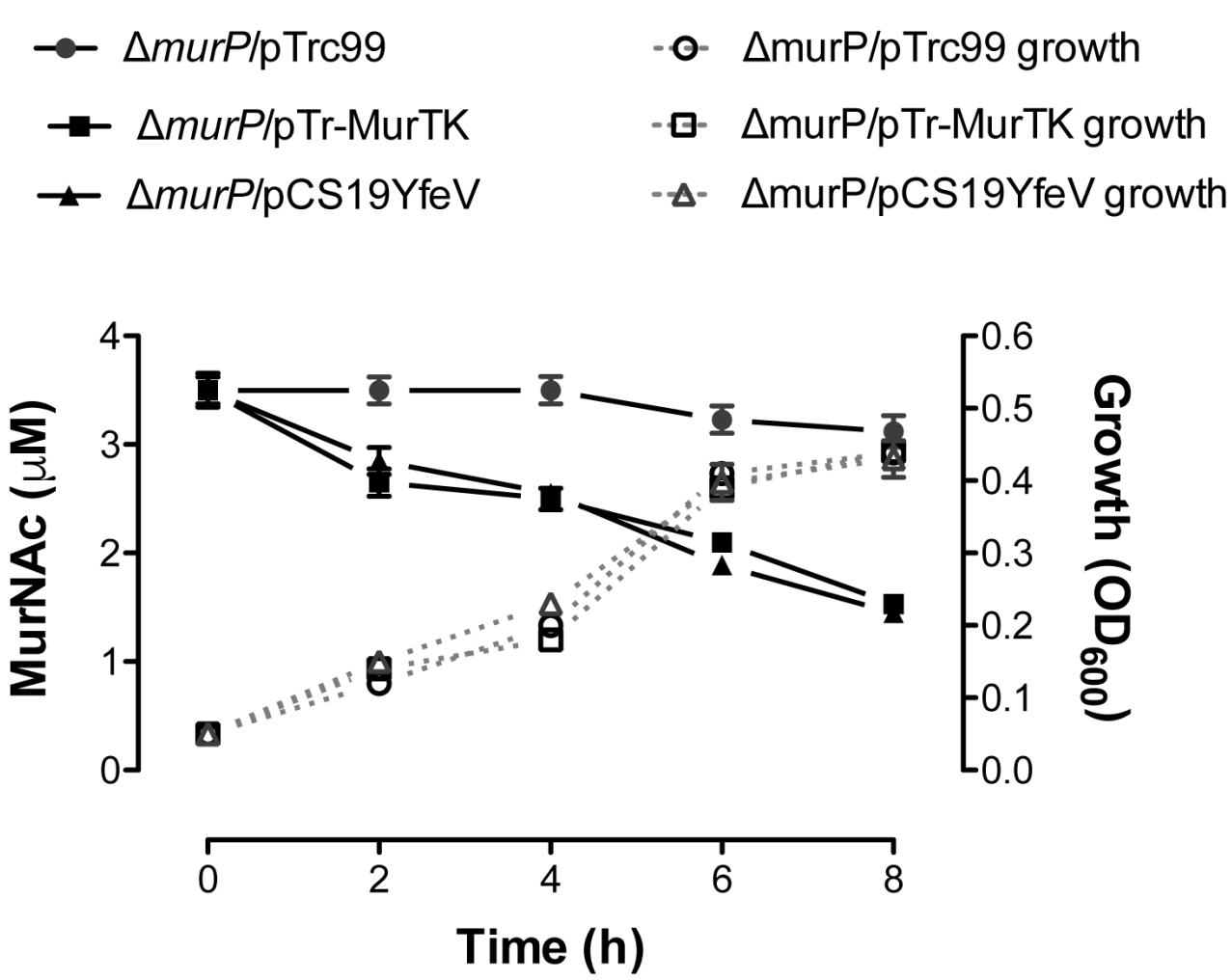
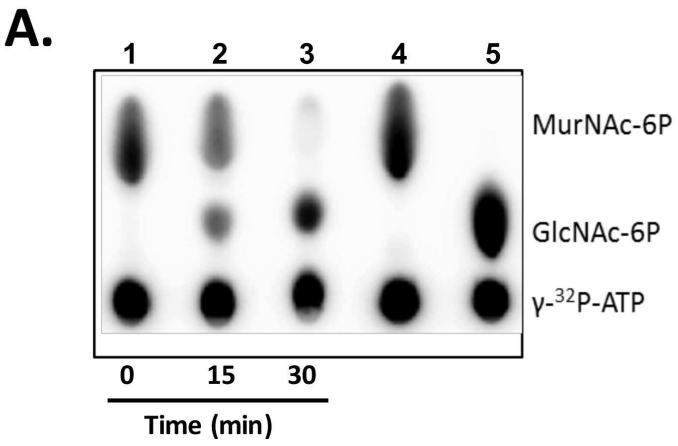
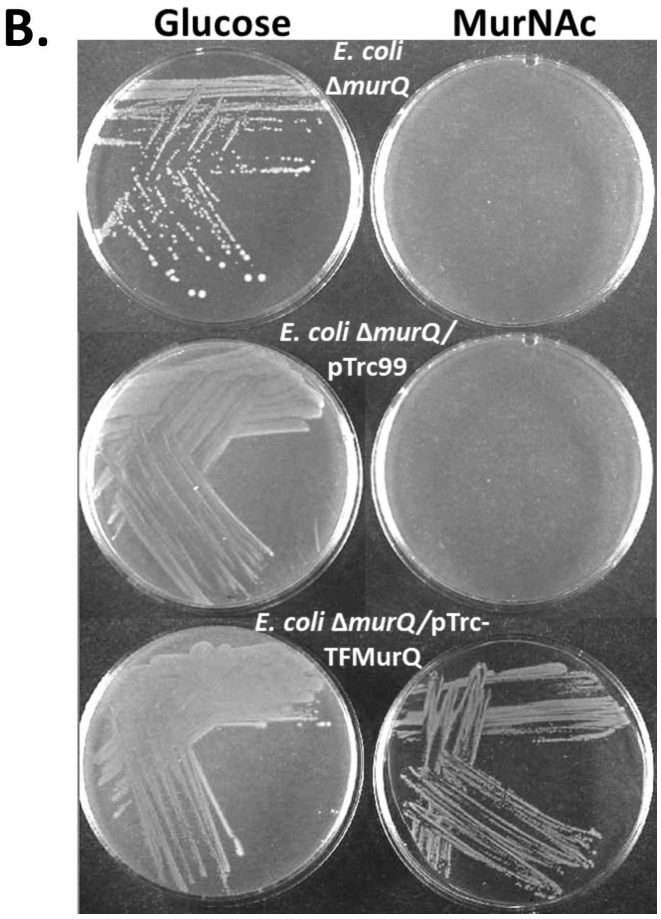
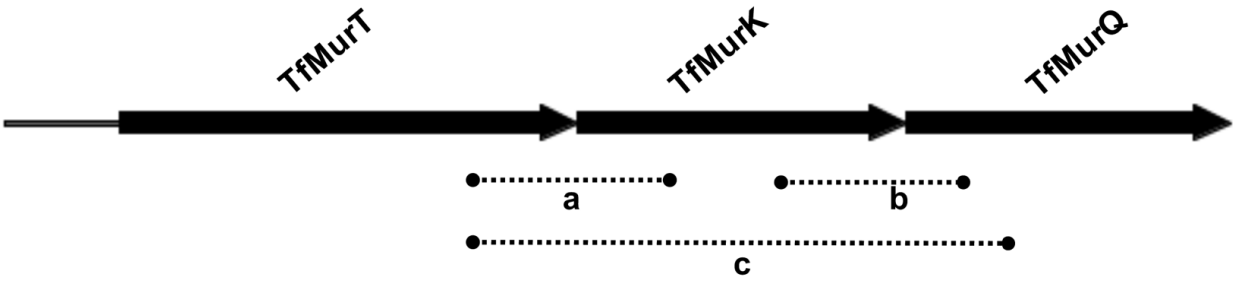








A.



B.