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RESEARCH ARTICLE

The secretome of *Thermococcus barophilus* in the presence of carbohydrates and the potential role of the TrmBL4 regulator

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Abstract

Global transcriptional regulators are crucial for supporting rapid adaptive responses in changing environments. In Thermococcales, the TrmB sugar-sensing regulator family is well represented but knowledge of the functional role/s of each of its members is limited. In this study, we examined the link between TrmBL4 and the degree of protein secretion in different sugar environments in the hyperthermophilic Archaeon *Thermococcus barophilus*. Although the absence of TrmBL4 did not induce any growth defects, proteomics analysis revealed different secretomes depending on the sugar and/or genetic contexts. Notably, 33 secreted proteins present in the supernatant were differentially detected. Some of these proteins are involved in sugar assimilation and transport, such as the protein encoded by *TERMP_01455* (cyclomaltodextrin glucanotransferase), whereas others have intracellular functions, such as the protein encoded by *TERMP_01556* (pyruvate: ferredoxin oxidoreductase Δ subunit). Then, using reverse transcription quantitative polymerase chain reaction experiments, we observed effective transcription regulation by TrmBL4 of the genes encoding at least two ABC-type transporters according to sugar availability.

INTRODUCTION

Thermococcus barophilus MP was the first true hyperthermophilic piezophile archaeon isolated from a deep-sea hydrothermal vent (Marteinsson et al., 1999). Previous investigations have shown that its adaptation to high hydrostatic pressure (HHP) is achieved by the modulation of the expression of different gene clusters involved in energy, amino acid, and carbohydrate metabolism (Vannier et al., 2015). Notably, the maltose/maltodextrin ABC transporter (MD system) *TERMP_(01653–01658)* involved in carbohydrate metabolism is one of the HHP-responsive gene clusters (Vannier et al., 2011). The expression of this MD system increases under high pressure, as does the expression of two other carbohydrate ABC

transporters, *TERMP_(00275–00281)* and *TERMP_(01836–01840)*; Vannier et al., 2015). Interestingly, the operon *TERMP_(00275–00281)* includes a transcriptional regulator gene (*TERMP_00277*) that encodes TrmBL4 (TrmB-like protein 4), which is a family member of the global transcriptional regulator TrmB, known for its regulation of sugar metabolism. TrmB is especially known to be a repressor and/or activator of sugar ABC transporters and as a histone-like chromosomal stabilizer in Archaea (Gindner et al., 2014; Kim et al., 2016).

Proteins of the TrmB family (TrmB, TrmBL1, TrmBL2, TrmBL3, TrmBL4, and TrmBL5) are present in 13 groups of Euryarchaeota (Lee et al., 2008). *Pyrococcus furiosus* has been shown to possess the genes *trmB* (PF1743), *trmBL1* (PF0124), *trmBL2* (PF0496),

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and *trmBL3* (PF066; Lee et al., 2008). Transcription assays show that TrmB of *P. furiosus* acts as a transcriptional repressor for the trehalose/maltose transport system (TM system) with high DNA binding affinity and for the MD system with lower affinity; this inhibition can be removed by the addition of specific sugars (Lee et al., 2005). Although, the TrmBL1 of *P. furiosus* acts as a repressor of genes of the MD system and glycolytic enzymes, and as an activator of genes involved in glucogenic pathways, it is inactivated by maltose and maltotriose (Lee, Surma, et al., 2007). Only preliminary experiments have been done on TrmBL2, which appears to recognize TM and MD promoters and to play a role at the DNA level, clumping it into dense fibrous structures (Maruyama et al., 2011). The TrmB family performs a multiplicity of regulations by acting on the same promoters: at least three regulators, TrmB, TrmBL1, and TrmBL2, regulate gene expression at the level of the MD promoter in *P. furiosus* (Lee et al., 2008). Interestingly, this species can grow on carbohydrates such as cellobiose, maltose, and starch (Kengen et al., 1993; Schmid et al., 2013), which implies the secretion of enzymes such as amylopullulanase (Schmid et al., 2013) and an α -glycosidase (Constantino et al., 1990).

The *trmB* gene in *Thermococcus litoralis* was characterized as a repressor of the TM system (Lee et al., 2003). The role of the other regulators (TrmBL3, TrmBL4, and TrmBL5) in the Thermococcales (Gindner et al., 2014) remains unclear. *Thermococcus barophilus* MP, the first true hyperthermophilic piezophile archaeon isolated from a deep-sea hydrothermal vent (Marteinsson et al., 1999), possesses the *TERMP_01972* gene encoding TrmB, which might regulate the adjunct operon *TERMP_(01968–01973)*; TM system); TrmBL2 encoded by the *TERMP_00762* gene; TrmBL4 encoded by the *TERMP_00277* gene, which can regulate the operon *TERMP_(00278–00281)* in the vicinity and divergently oriented (Figure 1A); TrmBL1 encoded by the *TERMP_01652* gene, which could regulate the operon *TERMP_(01653–01658)*; MD system; Figure 1B); and TrmBL5 encoded by the *TERMP_01835* gene, which might be involved in the regulation of the *TERMP_(01835–01840)* cluster in *T. barophilus* (Figure 1C; Kim et al., 2016).

The binding sites of TrmB and TrmBL1 were identified in *P. furiosus* (TrmB: TACTN₃AGTA for the TM system and TACT for the MD system; TrmBL1: TATCACN₅GTGATA for the MD system) as was the binding site of TrmB in *T. litoralis* (TACTN₃AGTA for the TM system; Lee et al., 2005). In 2006, Van de Werken et al. identified a conserved palindromic sequence, the Thermococcales glycolytic motif (TGM), TATCACN₅GTGATA, present upstream of all genes involved in sugar metabolism in several Thermococcales species as well as in the promoter region of the

MD operon, but not in the TM operon (Van de Werken et al., 2006). It has been shown that TrmB preferentially recognizes the TGM sequence (Lee, Seitz, et al., 2007), while TrmBL1 target binding is not restricted in this way because it also interacts with non-TGM-containing sequences with a lower binding affinity (Lee, Seitz, et al., 2007). TrmBL2 has no noticeable pattern with respect to other TGM-containing promoters (Lee, Seitz, et al., 2007; Lee, Surma, et al., 2007). The binding site of TrmBL4, however, has not yet been studied.

Because little is known about the metabolism of sugar and its regulation in *T. barophilus*, we investigated the potential role of TrmBL4 through a reverse genetic approach in which its gene locus (*TERMP_00277*) was deleted. We then analysed the global secreted proteins, known as the 'secretome' of the wild-type (WT) and mutant strains at 0.1 MPa in the presence of different carbohydrates. This made it possible, using a liquid chromatography with tandem mass spectrometry (LC-MS/MS) proteomic approach, to reveal different secretomes linked to the presence or absence of TrmBL4. Finally, we assessed the potential of TrmBL4 to regulate genes by performing reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiments on target genes involved in *T. barophilus* sugar metabolism.

EXPERIMENTAL PROCEDURES

Construction of mutants: Pop-in/Pop-out technique

The *TERMP_00277* locus (TrmBL4) was deleted by the Pop-in/Pop-out method to generate the *T. barophilus* Δ *trmBL4* strain following the protocol previously published in Birien et al. (2018) and Thiel et al. (2014) (Table 1).

Culture medium and growth experiments with sugar

Cells of *T. barophilus* WT *Tb* Δ 517 (UBOCC-M-3300; Birien et al., 2018) and the derivative mutant *Tb* Δ 517 Δ *trmBL4* (UBOCC-M-3321) were cultivated under anaerobic conditions in Thermococcales rich medium (TRM; Zeng et al., 2009) at a 1 \times or 1.1 \times concentration (when sugars were added). The medium was supplemented with different sugar sources: maltose (maltose monohydrate, Sigma Lot no. SLCC3973), maltodextrin (maltodextrin, Sigma Lot no. MKCG8957), or pectin (pectin from citrus, Sigma Lot no. 97H0580) at a final concentration 0.5 g/L. The medium was then transferred into 50 mL penicillin vials with or without elemental sulphur (0.25 g/L), which acts as a terminal

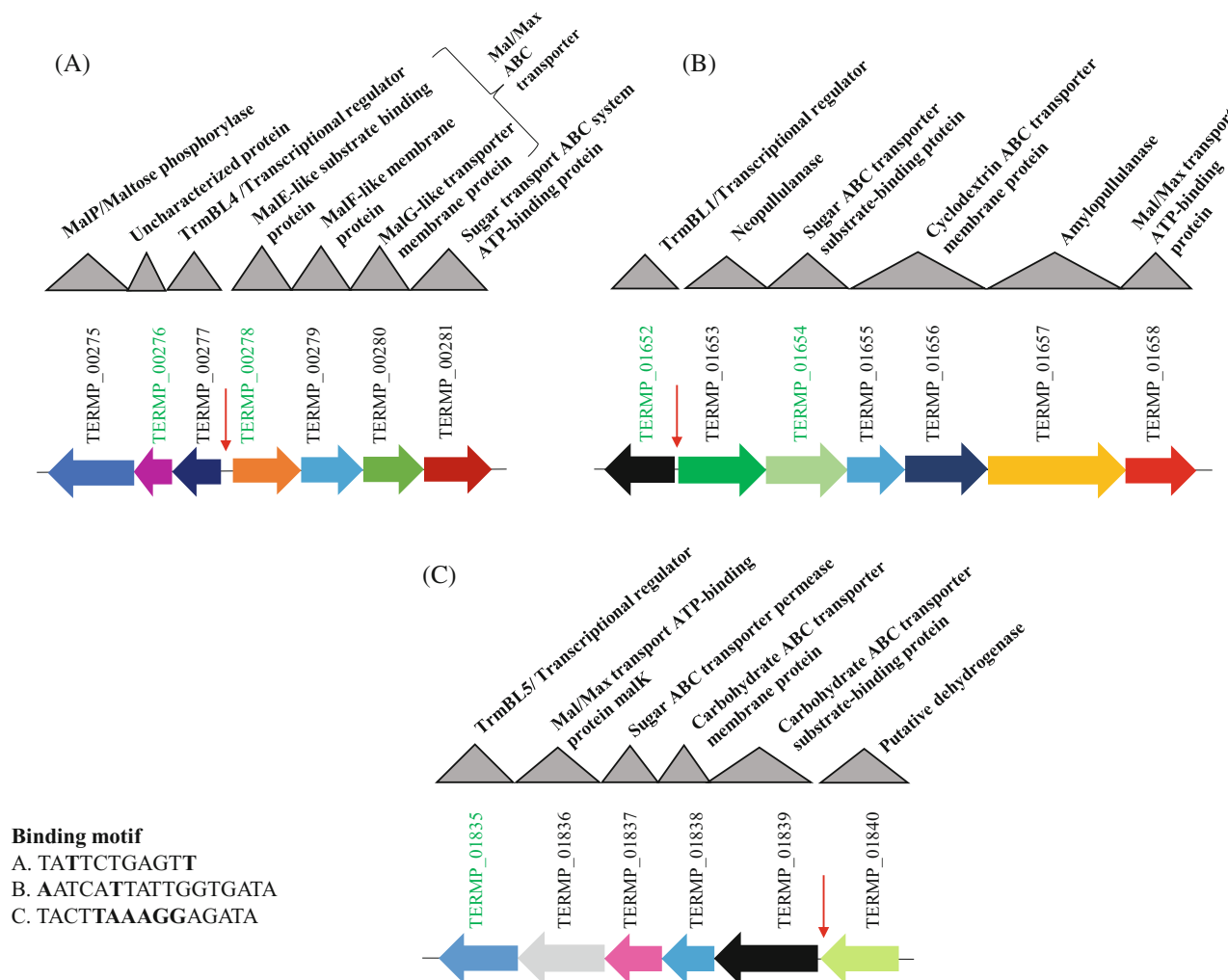


FIGURE 1 The organization of three clusters encoding genes involved in sugar metabolism and upregulated by high hydrostatic pressure in *Thermococcus barophilus*. (A) The *TERMP_(00275–00281)* cluster. (B) The *TERMP_(01652–01658)* cluster (MD system). (C) The *TERMP_(01835–01840)* cluster. The genes of interest representative of the target clusters for the reverse transcription quantitative polymerase chain reaction are highlighted in green. Red arrows indicate the TrmBL4 binding site and bold letters in the corresponding binding motifs indicate the non-conserved bases. Mal/Max indicates maltose/maltodextrin.

electron acceptor that enhances the growth of *Thermococcales* (Marteinsson et al., 1997). The cells were cultured at 85°C under P_{atm} . To determine growth in each treatment, cells were counted using a Thoma counting chamber (0.01 mm depth, Weber, England) at $\times 100$ magnification (Olympus BX40).

Recovery and concentration of extracellular proteins by ultrafiltration

To recover the secretome, the WT, and the mutant cells were cultured in 1-L bottles with 330 mL TRM medium with and without sugars and then incubated at 85°C under P_{atm} with shaking at 200 rpm. As noted above, each of the three sources of sugar used was added to give a final concentration of 0.5 g/L except for the pectin, for which this was 0.15 g/L, because pectin

interferes with the good separation and visualization of proteins. This minimum concentration of pectin was determined after several kinetic studies. For this, growth tracking of the cells was carried out in TRM medium in the presence of different concentrations of pectin (0.1, 0.15, 0.2, 0.25, 0.3, and 0.5 g/L) at 85°C (data not shown). Sulphur was added (0.25 g/L) to each treatment to enhance cell growth and thus obtain a sufficient cell concentration to study the secretome of the cells. The protocol for the concentration of extracellular proteins was modified from Schmid et al. (2013). The WT and mutant derivative cultures were collected at the late exponential phase ($1–2 \times 10^8$ cells/mL) and transferred to 450-mL centrifuge beakers to be centrifuged for 5 min at $200 \times g$, 4°C, to remove the rest of the sulphur from the medium. The media were transferred to new beakers for further centrifugation for 1 h at $8000 \times g$, 4°C. The supernatants were then filtered

TABLE 1 The list of specific primers used to delete the *trmBL4* gene using the Pop-in/Pop-out technique and to target genes of interest using the RT-qPCR technique.

TrmBL4 primer	Primer sequence
KpnI-D-TrmBL4 1 Fwd	5'AAAAAAGGTACCGCTATCAGAGTTTTAGAGTAGCATCTTCAATG 3'
D TrmBL4 1 Rev	5' TGCAGTGAGGGGTGGTTAATAAAAGGAGGGATCTCCTTGAAAGCA 3'
D TrmBL4 2 Fwd	5'GGAGATCCCTCCTTTTATTAACCACCCCTCACTGCATTATATTCT 3'
BglII-D TrmBL4 2 Rev	5'AAAAAAGATCTATCTCCAAGCCATCCAGCACCGACA 3'
Target gene	Primer sequence
<i>TERMP_00276</i> Fwd	5'ATTGCCTCATTGTTAATGCCGATTCTCC 3'
<i>TERMP_00276</i> Rev	5' TTAATTATGTCTCCGGTAAGCCGCG 3'
<i>TERMP_00278</i> Fwd	5' CCACCTACAACAGTGACAACAACAAA 3'
<i>TERMP_00278</i> Rev	5' GGGGAATTACCTCATATTTAACGCCGA 3'
<i>TERMP_01652</i> Fwd	5' GCTTAAGCAGGGTTATCAGCGTTCT 3'
<i>TERMP_01652</i> Rev	5' TTGGAGCCCTTCCAAGGGATAGA 3'
<i>TERMP_01654</i> Fwd	5 GGAGGCGGAACAACCACTC 3'
<i>TERMP_01654</i> Rev	5' GATTCAAAAACCTGCGCCTCATTTG 3'
<i>TERMP_01835</i> Fwd	5' CCTCAATCTCTACAGGCTCCTTGC 3'
<i>TERMP_01835</i> Rev	5' TTGCTGTTTCATGTTTGACAGGTACTTCT 3'
<i>TERMP_00342 (pcna)</i> Fwd	5' GCATGAGGGCAATGGATCCAA 3'
<i>TERMP_00342 (pcna)</i> Rev	5' GGGTTACCTCAAGGAAGTTCTCCTCA 3'
<i>TERMP_00128 (30S)</i> Fwd	5' GGCATAAACTTTGCCACGATGGTG 3'
<i>TERMP_00128 (30S)</i> Rev	5' TGCACCAATGAGGTGCATGTC 3'

Note: *TERMP_00276* and *TERMP_00278* representative of the *TERMP_(00275–00281)* cluster; *TERMP_01652* representative of the *TERMP_(01652–01658)* cluster; *TERMP_01654* representative of *TERMP_(01652–01658)*; *TERMP_01835* representative of *TERMP_(01835–01840)*; *TERMP_00342* and *TERMP_00128* used as control genes.

Abbreviation: RT-qPCR, reverse transcription quantitative polymerase chain reaction.

on sterile filters (0.22- μ m pore size) to remove cell remains. The proteins in the supernatant fractions (350 mL) were concentrated by two ultrafiltration procedures; first, by using an Amicon Ultra 15 10 K ultrafiltration unit following the manufacturer's procedure (Merck Millipore-Amicon® Ultra-15 10 K Centrifugal Filter Devices) to obtain a final volume of 100 μ L. A minimum molecular weight of 10 kDa was chosen after research carried out in silico showed that most of the proteins likely to be secreted by *T. barophilus* would be larger than 10 kDa. The second ultrafiltration procedure was performed using an Amicon ultra 2-mL ultrafiltration unit. This filtration reduced the volume to 70 μ L. To properly visualize proteins on an SDS-PAGE gel, the concentration of the proteins present in the supernatant must be 1 mg/mL; therefore, the protein concentration in each treatment was determined by Bradford assay using a standard range of known concentrations (Schmid et al., 2013). Additionally, to correctly filter the potentially secreted proteins, the cytosolic proteins were also concentrated. For this, the recovered cell pellet was centrifuged at 8000 \times *g* for 30 min at 4°C. Cell lysis was then performed by sonication by pulses of 0.5/0.5 s for 5 min at 4°C. Afterward, the sonicate was centrifuged at 10,000 \times *g* for 30 min. The same protocol was applied to concentrate the cytosolic proteins used as a control.

SDS-PAGE and identification of proteins by MS

To visualize and separate proteins, the concentrated proteins from the supernatants of *T. barophilus* WT and mutant strains grown in the presence of sugars (maltose, maltodextrin, or pectin), were applied to a 4%–20% Tris-Glycine Mini-Protean gel (Bio-Rad, Hercules, California). The deposited protein volume ranged from 10 to 20 μ L, containing 1 mg/mL of proteins, although the final deposited volume needed to be the same for all tested conditions. To cater for this, phosphate-buffered saline was used as an equilibration buffer and 4 \times XT sample buffer (Bio-Rad no. 1610791) was used as a stain. The gel was stained with Coomassie Brilliant Blue R250 (0.2% wt/vol, 40% vol/vol isopropanol, and 7% vol/vol acetic acid). The gel was then destained with a mixture of 300 mL 97% ethanol and 100 mL glacial acetic acid in a total volume of 1 L. Gel lanes were sliced into pieces and sent to the PAPPISO platform (*Plateforme d'Analyse Protéomique de Paris Sud Ouest*) for LC–MS/MS analysis on a 1D gel track (shotgun); the cytosolic concentrated proteins were rather used as a control for the secretion process. The gel slices were digested with 200 ng trypsin, then the samples were dispersed in 20 μ L loading buffer and 4 μ L were injected on a Fusion Lumos Orbitrap Tribrid mass

spectrometer. Quantitation of the proteins in all samples was performed using a Nanodrop 8000. Data processing was done with the X!TandemPipeline (0.4.40).

Analysis of secreted proteins

To properly filter the potentially secreted proteins, two procedures were used. The first of these was the detection of signal peptides *in silico* with the OutCyte 1.0 program (Zhao et al., 2019). The second was the determination of the probability that a protein is secreted or not based on the results of MS analysis of the cytosolic proteins used as a control of secretion. In the calculations, detailed in Data S2, the determination of the secretion probability is based on the ratio of the amount of the protein in the supernatant to its presence in the cytosol while the cells are grown in the presence of the three tested sugars. Consequently, the secretion probability for each protein was determined and a heatmap was constructed based on a colorimetric scale showing the difference of normalized spectra (at 10,000/sample), which intuitively indicates the proteins that are the most abundant in the secretome of the tested samples. Hypothetical proteins with putative domain annotations were analysed using the Pfam 34.0 (Mistry et al., 2021) wrapper around the HMMER package, which analyses biosequences using hidden Markov models (Durbin et al., 1998), and the UCSC Archaeal Genome Browser database (Schneider et al., 2006). In the latter, the basic gene annotation is derived from the NCBI GenBank/RefSeq entries, with conservation overlays of sequences across multiple species and nucleotide/protein motifs, as well as non-coding RNA and operon predictions.

The hierarchically clustered heatmap was constructed with the cluster map API of the statistical data visualization Python library *seaborn* (Waskom, 2021). The weighted linkage method was used for calculating clusters.

Sample RNA extraction and complementary DNA synthesis

RNA was extracted with TRIzol. For this, the strains were pre-cultured in a 20 mL TRM culture medium in the presence of sulphur, with and without maltose. WT and $\Delta trmBL4$ were inoculated at a rate of 2×10^6 cells/mL of fresh *T. barophilus* pre-culture, cultured for 6 h at 85°C at P_{atm} , and harvested at the mid-log phase by centrifugation at $5000 \times g$ for 6 min at 4°C. The pellet was re-suspended in 1 mL of Trizol and then transferred into 2 mL RNase-free tubes. Then, 200 μ L chloroform was added, followed by shaking by hand. The mixture was left for 3 min at room temperature. 15-min centrifugation was carried out at $12,000 \times g$, 4°C.

Afterward, the aqueous phase was placed in a 1.5 mL RNase-free Eppendorf. To precipitate the RNA, 500 μ L of isopropanol was added and the mixture incubated for 5 min at room temperature. The pellet was washed twice with 70% RNase-free ethanol followed by centrifugation for 15 min at $12,000 \times g$. After successive washings, the supernatant was removed, the residual ethanol dried out, and the RNA dissolved in water. Residual DNA was removed by DNase treatment (3 U of Turbo™ DNase, Invitrogen, according to the manufacturer's instructions). The quantity of extracted RNA was determined using a Nanodrop 8000 (Thermo Fisher Scientific Inc.) and it was stored at -80°C until use. The reverse transcriptions were performed using the iScript Reverse Transcription Supermix Kit for RT-qPCR (Bio-Rad) to obtain complementary DNA (cDNA) concentration of ~ 2.5 ng/ μ L. cDNAs were amplified (5 min at 25°C, 20 min at 46°C, and 1 min at 95°C).

Reverse transcription quantitative polymerase chain reaction

Quantitative PCR analysis was carried out on three clusters of genes, each with a representative gene, and primers were designed using SnapGene software (Figure 1 and Table 1). qPCR reactions with *pcna* and the *30S* gene were used as an internal control. Three biological replicates were performed for each treatment. Data were recorded and processed using Bio-Rad CFX Maestro software. The analysis was performed using the $\Delta\Delta\text{Ct}$ method to calculate expression changes compared with the control (Livak & Schmittgen, 2001; Schmittgen & Zakrajsek, 2000). The control gene corresponds to a gene in the genome expressed under all growth conditions and is, therefore, unregulated. This means that the expression of this gene should be relatively stable under all the studied conditions.

RESULTS

Thermococcus barophilus growth enhancement by carbohydrates

To test the ability of *T. barophilus* to metabolize carbohydrates, we assessed its growth kinetics in TRM medium with or without the sugars maltose (MAL), maltodextrin (MAX), or pectin (PEC), each at a final concentration of 0.5 g/L. We compared the growth of the derivative mutant $\Delta trmBL4$ strain against the WT to explore the potential role of the uncharacterized TrmB-like family regulator. Knowing the critical role of sulphur in Thermococcales growth (Bertoldo & Antranikian, 2006), the cultures were also grown in the presence (Figure 2AB) or absence | (Figure 2C,D) of elemental sulphur (0.25 g/L).

Our results show that growth is slightly enhanced in the presence of any sugar source, with an increase in the number of the WT cells, reaching a cell yield of 1.6×10^8 cells/mL at stationary phase (Figure 2A, blue dots, with sulphur), and a growth rate of 0.79 h^{-1} with maltose (Figure 2E). This compares with 8×10^7 cells/mL (Figure 2A, red dots, with sulphur) and a growth rate of 0.59 h^{-1} in the absence of sugars. In the case of the $\Delta trmBL4$ cells, the cell yield reached 2.5×10^8 cells/mL at stationary phase (Figure 2B, blue dots, with sulphur), with a growth rate of 0.96 h^{-1} with maltose (Figure 2E), compared with 9.1×10^7 cells/mL (Figure 2B, black dots, with sulphur) and a growth rate of 0.74 h^{-1} without any sugar (Figure 2E). The difference in cell concentration between the treatments with and without sugars was more pronounced when sulphur was absent from the medium. Cell growth in the presence of sugars reached 4.3×10^7 and 7×10^7 cells/mL, with growth rates of 0.42 and 0.56 h^{-1} , in WT and $\Delta trmBL4$ cells, respectively, in the presence of maltodextrin (Figure 2C,D, green dots, and Figure 2E without sulphur) compared with $\sim 6 \times 10^6$ cells/mL for both strains in the absence of sugars (0.2 h^{-1} growth rate; Figure 2C,D, red/black dots and Figure 2E without sulphur). Furthermore, a boost in the growth of the mutant cells compared with the WT strain was detected in the presence of maltose and sulphur (blue dots on Figure 2A,B, respectively), as the cell yields reached 2.5×10^8 cells/mL in $\Delta trmBL4$ at stationary phase but only 1.6×10^8 cells/mL in WT. These improvements in cell growth suggest the secretion of enzymes capable of degrading carbohydrates in the culture medium.

Extracellular proteins detected in the supernatant

Cells from the WT and $\Delta trmBL4$ strains were grown in TRM medium supplemented with carbohydrates (maltose 0.5 g/L , maltodextrin 0.5 g/L , or pectin 0.15 g/L), in the presence of sulphur (0.25 g/L), to obtain sufficient proteins for testing and analysis. Cells were harvested at the end of the exponential growth phase (10 h) and their supernatant fractions were collected. The concentrated proteins were separated by 1D-gel electrophoresis (Figure 3), cut into slices, and sent to the PAPPISO platform for MS analysis, as were the cytosolic proteins used as a control. There, the proteins were analysed using high-resolution LC-MS/MS. A few additional proteins were detected on the gel in the case where the WT and mutant cells were cultured in the presence of sugars (indicated on the gel, Figure 3). For example, a protein of 44 kDa molecular weight was present in the secretome of the WT cells cultured in the presence of any sugar but absent when no sugars were provided (Figure 3, Band III). Another protein, 120 kDa , was only detected in the secretome of the WT cells in the

presence of maltodextrin (Figure 3, Band II). In addition, a protein of 87 kDa was detected in the secretome of the mutant cells in the presence of sugars but was absent when none were provided (Figure 3, Band V). Despite the decrease in the concentration of pectin (0.15 g/L instead of 0.5 g/L) for the proteomic analysis, as explained in the experimental procedures section, technical issues were encountered when preparing proteins from the supernatant of the WT cells grown in the presence of pectin (data not shown). Consequently, the secretome of $\Delta trmBL4$ cells in the presence of pectin was not studied. To obtain a description of the dataset generated by the LC-MS/MS, a hierarchical clustering heatmap was constructed based on the values, showing the difference of normalized spectra (at $10,000/\text{sample}$). This method reveals which of the secreted proteins are the most abundant in the secretome of the tested samples (Figure 4 and Data S3). The detected cytosolic proteins were removed from the overall data (Data S1) to focus on the secretome. A total of at least 33 proteins were secreted in the supernatant of the WT and mutant cells cultured in the presence of sugars (Figure 4, Table 2, and Data S2). The corresponding heatmap highlights a WT-mutant dichotomy. Figure 4 shows the mutant cells on the left and the WT cells on the right, cultivated in the presence or absence of sugars. With the added carbohydrates, we see a clear shift in the secreted proteins of the mutant cells, with only some slight differences between maltose and maltodextrin. This did not happen with the WT cells, where notable differences could be observed, for example, the protein encoded by *TERMP_01807* (S-layer domain) was only expressed in the WT cells with maltodextrin. Interestingly, no common effect was detected between the three sugars, which could be because pectin may induce another secretome closer to the one that appears without sugar than to those with maltose or maltodextrin.

Among the proteins of the secretome, several were repressed in the mutant cells under particular conditions. These included a protein encoded by *TERMP_01556*, a pyruvate ferredoxin oxidoreductase Δ -subunit catalysing one of the final steps in carbohydrate fermentation (Blamey & Adams, 1993), which was found to be repressed in the mutant cells regardless of sugar availability. Two hypothetical proteins, respectively encoded by *TERMP_01372* and *TERMP_02026*, were also repressed in the mutant. Conversely, we detected an overexpression of some proteins in the mutant strain. These included the protein encoded by *TERMP_02130*, which was expressed slightly more in the mutant cells than in the WT cells; and the protein encoded by *TERMP_00597*, hypothetically coding for an alpha-galactosidase NEW3 domain, whose expression was higher in the mutant cells cultured without sugars. Additionally, the proteins encoded by

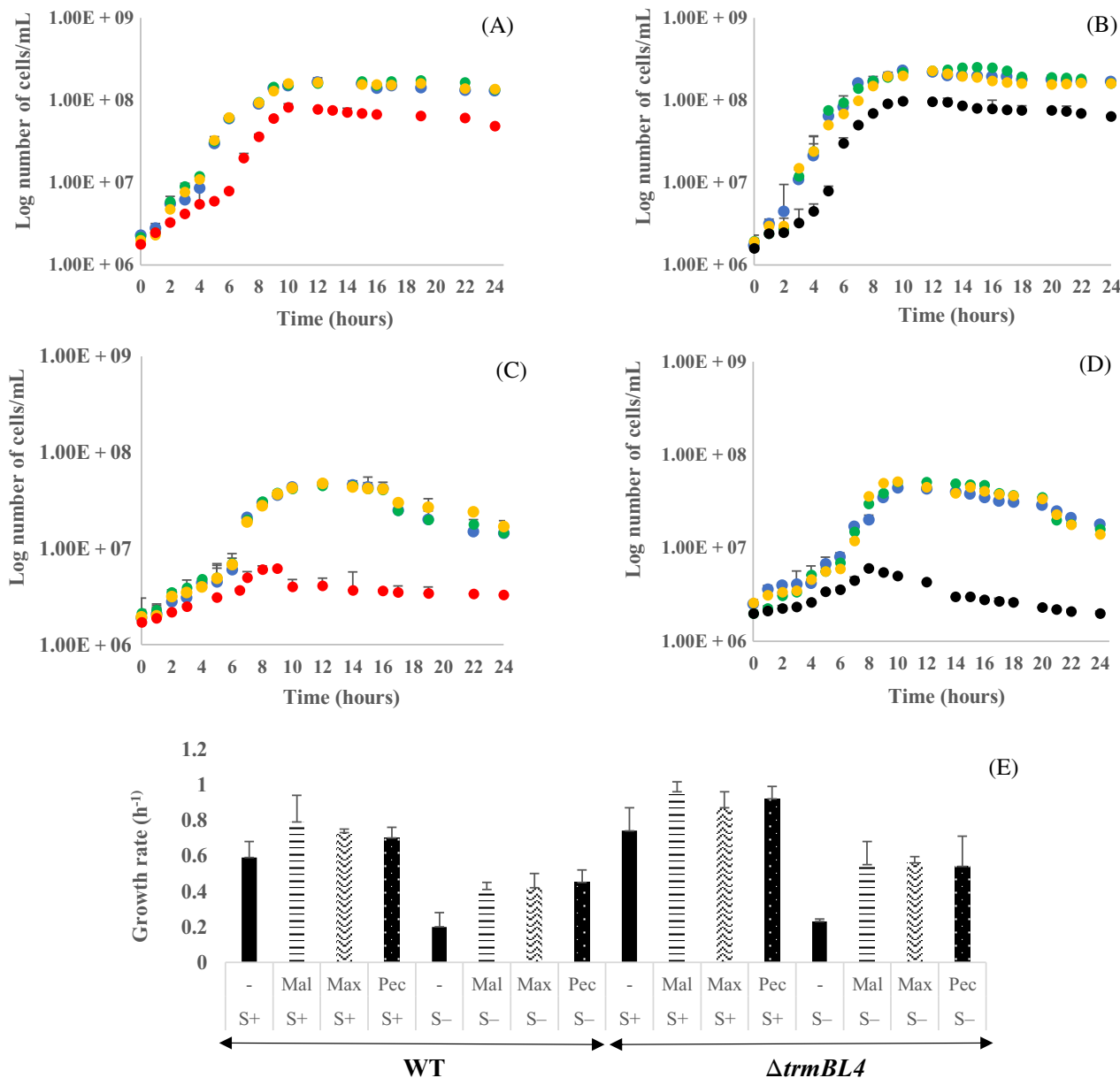


FIGURE 2 Tracking the growth of the wild-type (WT) and the $\Delta trmBL4$ cells with or without sugars in the presence and absence of sulphur at 0.1 MPa. WT cells cultured without sugars (●); $\Delta trmBL4$ cells cultured without sugars (●); cells cultured in the presence of maltose (●), maltodextrin (●), or pectin (●). (A) The growth of the WT cells in the presence and absence of sugars in the presence of sulphur. (B) The growth of $\Delta trmBL4$ cells in the presence and absence of sugars in the presence of sulphur. (C) The growth of the WT cells in the presence and absence of sugars in the absence of sulphur. (D) The growth of $\Delta trmBL4$ cells in the presence and absence of sugars in the absence of sulphur. (E) Comparison of the growth rate of the WT and $\Delta trmBL4$ cells in the presence of maltose (Mal), maltodextrin (Max), and pectin (Pec), in the presence and absence of sulphur at 0.1 MPa. A different pattern is used for each type of sugar for both WT and $\Delta trmBL4$ cells. A solid fill is used where no sugar was added. The growth rate, which is the inverse of generation time (G), represents the number of generations per unit of time in an exponentially growing culture. It is equal to $1/G$, expressed in inverse units of time (h^{-1}). The generation time (G) represents the time necessary for the doubling of a population in the exponential phase. Error bars represent standard deviations obtained from at least three biological replicates with three technical replicates each.

TERMP_00275, *TERMP_00281*, and *TERMP_00278*, which are part of the HHP-responsive cluster and close to the *TrmBL4* regulator (Vannier et al., 2015), were also overexpressed and only detected in the case of the mutant. However, the protein encoded by *TERMP_01657*, coding for an amylopullulanase that is part of the second HHP-responsive cluster *TERMP_*

(01653–01658; MD system), was overexpressed in both the WT and mutant secretomes in the presence of maltose and maltodextrin, whereas the other proteins of this operon were only slightly overexpressed in the mutant in the presence of sugars (Data S1). Regarding the *TERMP_(01836–01838)* operon, no expression was detected.

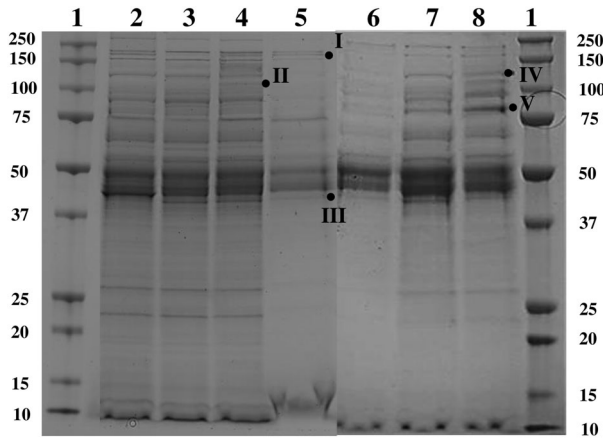


FIGURE 3 A representative Coomassie blue stained SDS-PAGE gel showing concentrated proteins from *Thermococcus barophilus* wild-type (WT) and $\Delta trmBL4$ cell culture supernatants.

1. PageRuler™ Prestained Protein Ladder; molecular weights are indicated in kDa; 1 mg/mL of proteins from each culture supernatant was loaded. 2. WT culture in the absence of sugars. 3. WT culture in the presence of maltose. 4. WT culture in the presence of maltodextrin. 5. WT culture in the presence of pectin. 6. $\Delta trmBL4$ culture in the absence of sugars. 7. $\Delta trmBL4$ culture in the presence of maltose. 8. $\Delta trmBL4$ culture in the presence of maltodextrin. A few additional bands detected in the presence of sugars are indicated by Roman numerals.

Surprisingly, the proteins encoded by *TERMP_00360* and *TERMP_02126* were expressed in all tested conditions. The first of these, corresponding to DNA protection during starvation protein, is known to protect DNA during nutrient starvation and against oxidative DNA damage (Frenkiel-Krispin et al., 2001). The second corresponds to a vWFA domain-containing protein known to be involved in cell adhesion, signal transduction, extracellular matrix proteins, and integrin receptors (Whittaker & Hynes, 2002).

Furthermore, the expression of the hypothetical proteins encoded by *TERMP_01984*, *TERMP_01543*, and *TERMP_02023* was altered by both the mutation and the presence of sugars because they are expressed only in the WT cells in the absence of sugars. In contrast, we detected the presence of a cyclomaltodextrin glucanotransferase, encoded by *TERMP_01455*, whose expression is controlled by the presence of maltose and maltodextrin in the medium independently of the mutation.

In addition, we observed that the expression of putative RNA binding proteins was altered the proteins encoded by *TERMP_00403* and *TERMP_01500* were repressed in the mutant cells compared with the WT cells in the absence of sugars. This was also the case with the phosphoesterase encoded by *TERMP_00076*, whose expression was activated in the mutant cells by the addition of sugars. These proteins were found in the supernatant of the cells but had no detectable signal peptide.

Relative expression of genes in the presence and absence of sugars studied by RT-qPCR

To determine the potential consequences of the deletion of the *trmBL4* gene for the expression of the three HHP-responsive gene clusters involved in the metabolism of sugars in *T. barophilus*, we performed RT-qPCR experiments targeting some representative genes of clusters of interest determined by the results of the LC-MS/MS analysis and the transcriptomics data published by Vannier et al. (2015). These genes were *TERMP_00276* and *TERMP_00278*, representative of the *TERMP_(00278–00282)* cluster (Figure 1A); *TERMP_01652* and *TERMP_01654*, representative of the *TERMP_(01652–01658)* cluster (Figure 1B); and *TERMP_01835*, representative of the *TERMP_(01835–01840)* cluster (Figure 1C). The effects of the deletion of the *trmBL4* gene and of sugar addition were tested (Figure 5A,B, respectively). When comparing the results of $\Delta trmBL4$ and WT cells cultivated in the absence of maltose (Figure 5A), a repression of the expression of the representative genes of *TERMP_(00275–00281)* and *TERMP_(01652–01658)* was observed in the mutant strain, implying that *trmBL4* acts as an activator of the expression of the corresponding genes in the absence of maltose. Moreover, the results of WT cells cultivated in the presence or absence of maltose highlight repression of the same representative genes in the presence of maltose (*TERMP_00276*, *TERMP_00278*, *TERMP_01652*, and *TERMP_01654*). Comparison between the results of $\Delta trmBL4$ and WT showed that, in the presence of maltose, the expression of the representative genes of *TERMP_(00275–00281)* and *TERMP_(01652–01658)* was increased in the mutant strain, linking *TrmBL4* as a repressor of the gene expression. So far, the operon *TERMP_(00275–00282)* seemed to be regulated by *TrmBL4*. The deletion of the *trmBL4* gene once again allowed, in the presence of maltose, an increase in the relative expression of this operon (Figure 5B). Concerning the expression of *TERMP_01654*, the representative gene of the *TERMP_(01652–01658)* operon, the increase was smaller, probably because of a lower affinity of the *trmBL4* regulator on this operon. In contrast, the expression of *TERMP_01835*, the representative gene of the *TERMP_(01835–01840)* cluster, was not affected by *TrmBL4* and was only slightly impacted by the addition of sugar.

Identification of *TrmBL4* DNA binding domains

To check for a connection between *TrmBL4* and the genes that seem to be controlled by this regulator, an in silico screening for the *TrmB*-like binding motif was

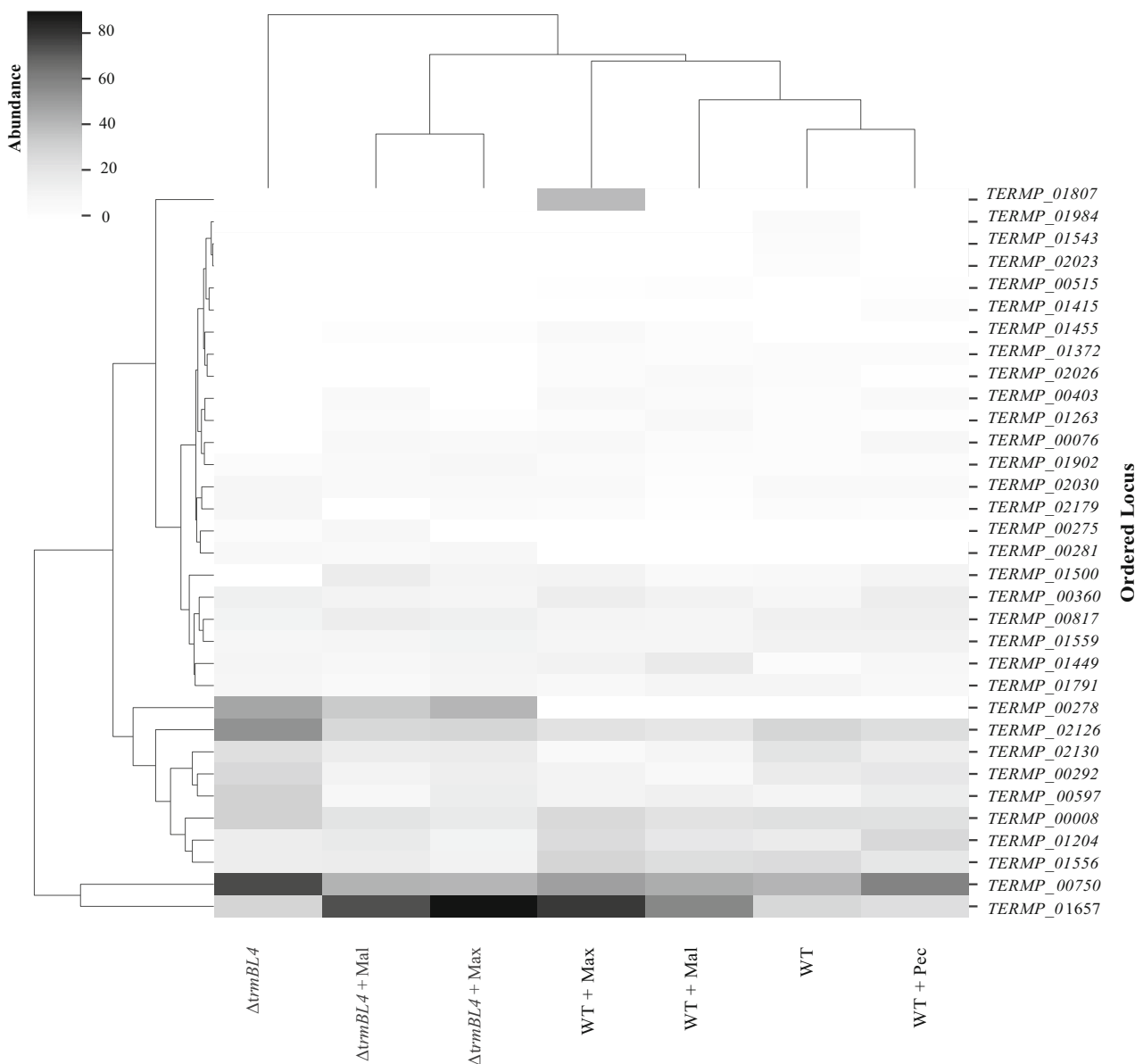


FIGURE 4 Hierarchical heat map for the visualization of the differential expression of the secreted proteins in each sample. The heat map was produced with the *seaborn* program using the liquid chromatography with tandem mass spectrometry data and shows only the secreted proteins (Data S3) from Data S1. The samples are shown in the columns and the ordered loci for the genes encoding the secreted proteins in the rows. Columns are centred, with relative abundance (the difference of normalized spectra) represented by the darkness of shading (white: lower abundance; black: higher abundance) of the secreted proteins in the tested conditions. Mal, Max, and Pec indicate maltose, maltodextrin, and pectin, respectively.

performed on the whole genome of *T. barophilus* based on the known binding motifs of TrmB and TrmBL1 discovered in other Thermococcales (Lee et al., 2008; Lee, Surma, et al., 2007). We detected a high degree of conservation between the binding motifs in *T. barophilus* compared with those known in *P. furiosus*. Concerning the *Pfu*-TrmB binding site, the same TACTN₃AGTA sequence was found in *T. barophilus* for the TM system, except for two nucleotides that differ from the *Pfu*-TrmBL1 binding site for the MD system (TATCACN₅GTGATA in *P. furiosus* and AATCATN₅GTGATA in *T. barophilus*).

A binding site (AATCATN₅GTGATA) was found upstream of *TERMP_01653* (MD system) at position 1,439,024–1,439,040. This binding motif includes the second half of the TGM sequence **GTGATA**. Moreover, the binding site (TACTN₃AGTA) was detected upstream of *TERMP_01968* (TM system) at the positions 1,762,048–1,762,068 and 1,762,230–1,762,240. Two additional binding sites (TATTCTGAGTT, pos. 232,136–232,146 and TACTN₆AGATA, pos. 1,624,822–1,624,836) were detected upstream of *TERMP_00278* and *TERMP_01840*. Three additional binding sites (TACTTAGTT, pos. 1,295,540–1,295,548; TACTGCTT,

TABLE 2 The list of secreted proteins identified in the supernatant of *Thermococcus barophilus* wild-type and $\Delta trmBL4$ cells grown on sugars.

Ordered locus	Found only in the supernatant	Secretion probability	N-terminal sequence ^a	Molecular weight	Description	Pfam/UCSC archaeal genome ^b	TrmBL4 binding motif ^c
TERMP_01807	Yes	0.5	Yes	101.5	S-layer domain		No
TERMP_01984	Yes	1	Yes	68.1	Lipoprotein		No
TERMP_01543	Yes	1	Yes	45.2	Uncharacterized protein	Eight-cysteine-cluster domain	No
TERMP_02023	Yes	1	Yes	22	Flagellin		Yes
TERMP_00515	Yes	0.5	Yes	70.5	S-layer_C domain-containing protein		No
TERMP_01415	Yes	0.5	Yes	71.6	Uncharacterized protein	Hypothetical protein	No
TERMP_01455	Yes	0.5	Yes	81	Cyclomaltodextrin glucanotransferase		No
TERMP_01372	Yes	1	Yes	56.4	Uncharacterized protein	Amylopullulanase	No
TERMP_02026	Yes	1	Yes	51	Uncharacterized protein	Hypothetical protein	No
TERMP_00403	Yes	1	No	8.9	RNA-binding protein		No
TERMP_01263	Yes	1	No	27.7	Metal-dependent hydrolase		No
TERMP_00076	Yes	1	No	18.8	Phosphoesterase		No
TERMP_01902	Yes	1	No	17.6	Peptidyl-prolyl cis-trans isomerase		No
TERMP_02030	Yes	1	No	22	GMP synthase		No
TERMP_02179	Yes	1	Yes	209.5	Peptidase_S8 domain-containing protein		No
TERMP_00275	Yes	0.5	No	86.8	Maltose phosphorylase		No
TERMP_00281	Yes	0.5	No	40.1	Sugar ABC transporter		No
TERMP_01500	Yes	1	No	8.2	RNA-binding protein		Yes
TERMP_00360	No	1	No	21.2	DNA protection during starvation protein		No
TERMP_00817	No	1	No	21.5	Thymidine kinase		No
TERMP_01559	No	1	No	12	Ketisovalerate oxidoreductase subunit		No
TERMP_01449	Yes	1	Yes	76.4	Dockerin domain-containing protein		No
TERMP_01791	No	1	Yes	48.5	S-layer-like array protein		No
TERMP_00278	Yes	0.5	Yes	50.9	MalE-like Mal/Max ABC transporter		No
TERMP_02126	No	1	Yes	179.8	vWFA domain-containing protein		No

(Continues)

TABLE 2 (Continued)

Ordered locus	Found only in the supernatant	Secretion probability	N-terminal signal sequence ^a	Molecular weight	Description	Pfam/UCSC archaeal genome ^b	TrmBL4 binding motif ^c
TERMP_02130	Yes	1	Yes	21.8	Methyltransferase	No	No
TERMP_00292	No	1	Yes	54.3	PEGA domain-containing protein	Domain of α -galactosidase	Yes
TERMP_00597	No	1	Yes	80.6	NPCBM_assoc domain-containing protein	Domain of α -galactosidase	No
TERMP_00008	No	1	No	38.5	Glycosidase	No	No
TERMP_01204	No	1	Yes	22.8	Flagellin	No	No
TERMP_01556	No	1	No	12	Pyruvate: ferredoxin oxidoreductase Δ subunit	No	No
TERMP_00750	No	1	Yes	76.3	CARDB domain-containing protein	No	No
TERMP_01657	No	1	Yes	153.6	Amylopullulanase	Yes	Yes

Note: The supernatant only represents the proteins found only in the supernatant, not in the cytosol; the secretion probability is calculated according to the equations in Data S2 and Mal/Max for maltose/maltodextrin.

^aPrediction of N-terminal signal sequence using the OutCyste 1.0 program (Zhao et al., 2019).

^bSignificant hit obtained after a search in Pfam 34.0 (Mistry et al., 2021) and the UCSC Archaeal Genome Browser database for hypothetical proteins (Schneider et al., 2006).

^cSearch for the TrmB-like binding motif performed in silico on the whole genome of *T. barophilus*, based on the known TrmB binding motifs in certain Thermococcales.

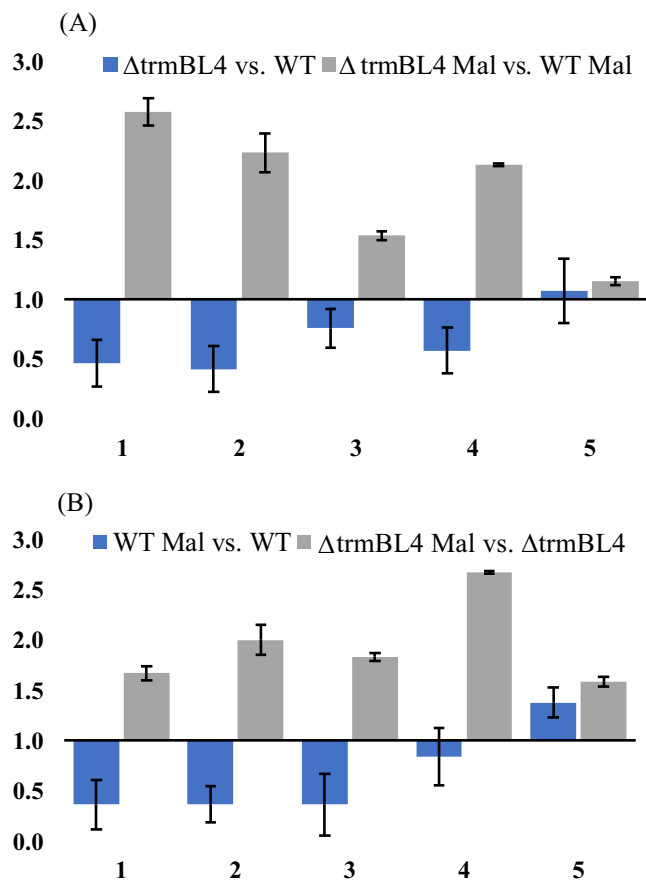


FIGURE 5 Comparison of the relative expression of the genes of interest representative of the target clusters under different conditions in the presence and absence of maltose, showing the effect of *trmBL4* gene mutation (A) and the effect of sugar (B). 1. and 2. *TERMP_00276* and *TERMP_00278* representative of the *TERMP_00275–00281* cluster; 3. *TERMP_01652* representative of the *TERMP_01652–01658* cluster; 4. *TERMP_01654* representative of *TERMP_01652–01658*; 5. *TERMP_01835* representative of *TERMP_01835–01840*.

pos. 1,806,176–1,806,183; and TACTCN₃CATA, pos. 246,815–246,826) were detected upstream of *TERMP_01500*, *TERMP_02023*, and *TERMP_00292*, respectively. In addition, a complete TGM motif was detected upstream of *TERMP_01654* (MD system) at position 1,441,219–1,441,235. Surprisingly, no TGM sequence was detected upstream of the *TERMP_00278–00281* operon or in other regulated glycolytic genes.

DISCUSSION

Little is known about the secretome of *T. barophilus*, which plays an important role in sugar uptake and metabolism. In this study, we made the first analysis of the carbohydrate metabolism in *T. barophilus* by studying its secretome when cultured in the presence of carbohydrates at P_{atm} using genetic, physiological, and

proteomics approaches. Our work represents the first step towards deciphering the organism's mechanism of adaptation to HHP. The regulation of sugar ABC transporters was assessed using deletion mutants of the transcriptional regulator of the TrmB family (*TrmBL4*) present in *T. barophilus*, which is known to affect the transcription of genes involved in sugar metabolism in Thermococcales. The effect of the deletion of the *trmBL4* gene was reflected by the growth enhancement of the mutant cells compared with the WT in the presence of sugars and these improvements were more pronounced in the absence of sulphur. Regardless of the mutation, the cells in a sulphur-free medium relied more on the sugars to grow and seemed to use sugars as a source of carbon for growth. These results are supported by studies showing that sulphur may play a role in the metabolism of sugars in Archaea (Adams et al., 2001).

The proteomic results highlight critical proteins of the *T. barophilus* secretome, which are probably at least regulated by the *trmBL4* gene product, and others might be impacted by cultivation under non-optimal growth pressure in *T. barophilus*. The STRING database search for protein–protein interactions (Szklarczyk et al., 2019) of the hypothetical amylopullulanase encoded by *TERMP_01372*, repressed in the mutant cells, revealed that the adjacent interacting proteins correspond to a secretion system. This probably indicates a new sugar-related secretion system under the control of the *trmBL4* gene product. Additionally, the interacting proteins encoded by *TERMP_02026* and *TERMP_02023*, repressed in the mutant cells, could have formed a cross-network regulating the cell shape and controlling the timing of the cell division in our tested conditions under the control of the *trmBL4* gene. This is likely because the adjacent interacting proteins, such as a tubulin cell division FtsZ-like protein (*TERMP_02021*), are involved in the control of cell division.

Surprisingly, the protein responsible for pyruvate conversion was expressed under all conditions, with just slight repression in the mutant cells. The presence of this enzyme can be explained either by the use of pyruvate to cope with non-optimal pressure or the activation of the conversion of pyruvate due to its accumulation in the cell. This pyruvate could thus be mobilized under stressful pressure conditions, as in the case of glutamate biosynthesis in *Thermococcus piezophilus* (Moalic et al., 2021).

Additionally, some proteins were found to be overexpressed in the mutant cells. These included a methyltransferase encoded by *TERMP_02130*, which may play a role in controlling the profile of gene expression impacted by the mutation. In addition, the search made in the STRING database (Szklarczyk et al., 2019, 2023) on another overexpressed key protein encoded by *TERMP_00597*, showed that the adjacent

interacting proteins correspond to ABC transporters, which may indicate their disposition in a potential operon involved in sugar transport.

Contrastingly, the expression of the DNA protection during starvation protein and the vWFA domain-containing protein in all tested conditions may indicate a stress response due to the alteration of nutrient uptake under non-optimal pressure conditions (0.1 MPa) and modulation of the cell signalling pathways at 0.1 MPa, respectively.

Both the *trmBL4* gene mutation and the carbohydrates were able, nonetheless, to repress the expression of the protein encoded by *TERMP_01543* as though, in the absence of sugars, the presence of the *trmBL4* gene activates its expression. Therefore, we took a closer look at this protein to decipher its role. *TERMP_01543* may contain an eight-cysteine-cluster domain that could play a role in the biofilm formation as in the fungus *Candida albicans* (Pérez et al. 2006). Indeed, it is known that Archaea biofilms are formed by extracellular polymeric substances composed of polysaccharides, proteins, and metal ions (Stoodley et al., 2002). Studies have indicated that biofilm formation in Archaea provides benefits to the cells, including protection against stressful growth conditions such as toxic chemicals and low or high pH (Koerdts et al., 2010; Megaw & Gilmore, 2017). The phenotypic shifts in the behaviour of Archaea caused by stressful growth conditions require many regulatory processes and lead to the development of biofilms (An & Parsek, 2007). Here, in *T. barophilus*, the mutation of the *trmBL4* gene, addition of sugars, and growth under non-optimal pressure conditions may have altered the biofilm formation by repressing genes exhibiting an enhanced ability to form biofilms. In *S. solfataricus*, the products of β -galactosidase and α -mannosidase can control the saccharide volume in the biofilm matrix (Koerdts et al., 2010).

Based on the proteomic results and the KEGG pathway database (Kanehisa, 2002), it is clear that maltose and maltodextrin share some enzymes and thus some common pathways, but pectin may not share the main assimilation systems with maltose and maltodextrin due to its complexity. Pectin is known to be degraded by specific pectinolytic enzymes with the corresponding transporters, as in the pectin degradation pathway in *Erwinia chrysanthemi* (now reclassified as *Dickeya dadantii*; Hugouvieux-Cotte-Pattat et al., 1996).

The S-layer domain protein encoded by *TERMP_01807*, which was only overexpressed in the presence of maltodextrin in the WT cells, is probably involved in a specific maltodextrin membrane transport system because its adjacent interacted protein encoded by *TERMP_01808* corresponds to a major facilitator superfamily transporter. Additionally, we think that the expression of proteins forming part of the S-layer domain is also pressure-dependent, highly



ghting their role in cell stabilization under sub-optimal pressure (0.1 MPa) since *TERMP_01807* was over-expressed at 0.1 versus 40 MPa (Vannier et al., 2015). This phenomenon was also observed in the gene expression patterns of *T. piezophilus* (Moalic et al., 2021).

Intriguingly, the expression of RNA-binding proteins requires the addition of sugars in the mutant cells, but not the WT cells. In other words, the presence of the *trmBL4* gene compensates for the absence of sugar. These proteins seem to have a role in regulating gene expression in our different tested conditions (Murina & Nikulin, 2011).

The presence of proteins in the supernatant of the cells devoid of a signal peptide can be explained by cell lysis, the limit of detection of the OutCyte program, or by them being classified as moonlighting proteins (Jeffery, 2003; Huberts & Van der Klei, 2010), which are uni-functional proteins able to operate on both intracellular and extracellular levels (Sengupta et al., 2008). Particularly in the case of Archaea, another possible explanation is the export of proteins via membrane vesicles (Ellen et al., 2009; Ellen et al., 2010; Soler et al., 2008). More work is needed to determine which of these possible explanations are valid.

Interestingly, the RT-qPCR results highlight a particular regulatory mechanism of the *trmBL4* gene product on some HHP-responsive gene clusters involved in carbohydrate metabolism. In the absence of maltose, TrmBL4 seems to activate gene expression but, in the presence of this sugar, it acts as a repressor of gene expression. In our case, sugars did not rescue transcription as seen in *Pfu*-TrmBL1 (Lee et al., 2008; Lee, Surma, et al., 2007). So, in the presence of maltose, the *trmBL4* regulator seems to act as a global transcriptional repressor of the *TERMP_(00275–00281)* cluster, with high binding affinity, and of the *TERMP_(01652–01658)* cluster (MD system), with lower affinity, defining it as a regulon. It should also be mentioned that the regulation of the expression of genes is not directly related to the composition of the secretome. The absence of TrmBL4 can have many more repercussions than those observed on the target genes tested by RT-qPCR, such as effects on other target genes, retro-control, and protein–protein interactions, which could all consecutively modify the secretome of *T. barophilus*.

In particular, the LC–MS/MS and RT-qPCR results may indicate synergy between TrmBL4 and TrmBL1, since each of these may regulate the MD operon but with different affinities. Indeed, the expression of the *trmBL1* gene encoded by *TERMP_01652* is increased in the mutant, as shown by the LC/MS–MS (Data S1) and the RT-qPCR results, as though compensating for the mutation of one of the regulators, the *trmBL4* gene. Consequently, we propose that these two members of the TrmB family are involved in a cross-regulatory network of sugar-sensing transcriptional regulators, as in

the case of *P. furiosus*, characterized by the multiplicity of regulators acting on the MD promoter (Lee et al., 2008). Thus, in the absence of maltose, the TrmBL4 regulator alone may repress its expression leading to the de-repression of the targeted genes, but repression is otherwise activated by maltose. This would mean that maltose stimulates the synthesis of TrmBL4, which would in turn repress the expression of genes encoding glycolytic enzymes. This emphasizes the important role of sugars in controlling transport and glycolysis. Moreover, TrmBL4 had no detectable effect on the *TERMP_(01835–01840)* operon. Interestingly, this operon was previously found to be overexpressed at high pressure (40 MPa; Vannier et al., 2015) but was not detected at 0.1 MPa.

The *in-silico* screening for TrmBL4 binding sites indicates a certain degree of conservation, showing a divergence linked to the specific evolution of TrmBL4 compared with other TrmB-like regulators. The latter need to be studied by footprint analysis on the promoter regions. The TrmBL4 target binding site also does not seem to be restricted to a TGM sequence because it is also involved in the regulation of genes with non-TGM-containing sequences.

CONCLUSION

This study presents the first description of sugar metabolism gene products in *T. barophilus* and the potential regulation of some of these by the *trmBL4* gene product at atmospheric pressure. Many valuable proteins have been identified that could be the subject of further molecular studies in the near future. Some of the gene clusters coding for these proteins appear to be under the control of TrmBL4, one of the five TrmB-like transcriptional regulators in the *T. barophilus* genome. It would be interesting to compare the composition of the secretome in the presence of sugars according to the presence or not of sulphur. It seems that, in a free sulphur medium, the sugars participate more actively in the energy metabolism and should therefore be more assimilated by the cells. Also, the regulation of the expression of the genes by TrmBL4 described in this study needs to be carried out at HHP because some genes of sugar metabolism are known to be responsive to these conditions. This is the first study of a TrmB family regulator in *T. barophilus*, opening up the possibility of a subsequent in-depth study to characterize the gene expression regulation mechanisms of sugar metabolism structurally, molecularly, genetically, and biochemically in relation to hydrostatic pressure.

AUTHOR CONTRIBUTIONS

Maria Batour: Methodology (equal); writing—original draft (equal). **Sebastien Laurent:** Data curation (equal); methodology (equal); writing—review and

editing (equal). **Yann Moalic**: Formal analysis (equal); methodology (equal); writing—review and editing (equal). **Hala Chamieh**: Supervision (supporting); writing—review and editing (equal). **Samir Taha**: Writing—review and editing (supporting). **Mohamed Jebbar**: Conceptualization (lead); funding acquisition (lead); project administration (lead); resources (lead); supervision (lead); validation (lead); writing—original draft (lead); writing—review and editing (lead).

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CONFLICT OF INTEREST STATEMENT


The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Datasets can be found in the Supplementary Data files.

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SUPPORTING INFORMATION

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