Changes in Transcriptome of *Yersinia pseudotuberculosis* IP32953 Grown at 3 and 28°C Detected by RNA Sequencing Shed Light on Cold Adaptation

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Yersinia pseudotuberculosis is a bacterium that not only survives, but also thrives, proliferates, and remains infective at cold-storage temperatures, making it an adept foodborne pathogen. We analyzed the differences in gene expression between *Y. pseudotuberculosis* IP32953 grown at 3 and 28°C to investigate which genes were significantly more expressed at low temperature at different phases of growth. We isolated and sequenced the RNA from six distinct corresponding growth points at both temperatures to also outline the expression patterns of the differentially expressed genes. Genes involved in motility, chemotaxis, phosphotransferase systems (PTS), and ATP-binding cassette (ABC) transporters of different nutrients such as fructose and mannose showed higher levels of transcripts at 3°C. At the beginning of growth, especially genes involved in securing nutrients, glycolysis, transcription, and translation were upregulated at 3°C. To thrive as well as it does at low temperature, *Y. pseudotuberculosis* seems to require certain cold shock proteins, especially those encoded by *yptb3585*, *yptb3586*, *yptb2414, yptb2950,* and *yptb1423*, and transcription factors, like Rho, IF-1, and RbfA, to maintain its protein synthesis. We also found that genes encoding RNA-helicases CsdA (*yptb0468*), RhlE (*yptb1214*), and DbpA (*yptb1652*), which unwind frozen secondary structures of nucleic acids with cold shock proteins, were significantly more expressed at 3°C, indicating that these RNA-helicases are important or even necessary during cold. Genes involved in excreting poisonous spermidine and acquiring compatible solute glycine betaine, by either uptake or biosynthesis, showed higher levels of transcripts at low temperatures. This is the first finding of a strong connection between the aforementioned genes and the cold adaptation of *Y. pseudotuberculosis*. Understanding the mechanisms behind the cold adaptation of *Y. pseudotuberculosis* is crucial for controlling its growth during cold storage of food, and will also shed light on microbial cold adaptation in general.

Keywords: cold stress, stress tolerance, compatible solute, RNA helicase, cold shock protein, transcription factor

INTRODUCTION

Yersinia pseudotuberculosis is an enteropathogenic bacterium that causes the foodborne infection yersiniosis. Although it has an optimum growth temperature of around 28°C, *Y. pseudotuberculosis* can grow and multiply at cold-storage temperatures, even as low as 0°C (Keto-Timonen et al., 2018). Long storage at low temperature favors the growth of psychrotrophs like *Y. pseudotuberculosis*, as they can proliferate without much competition. Outbreaks of *Y. pseudotuberculosis* have been associated with vegetables, raw milk, and drinking untreated water (Sato and Komazawa, 1991; Nuorti et al., 2004; Rimhanen-Finne et al., 2009; Pärn et al., 2015). In order to survive the various ecological niches through which it could enter the food chain, *Y. pseudotuberculosis* carries many tools in its genome, such as operons for the transport of substrates more abundant in plants and soil than animal tissue, as well as type VI secretion systems (Jaakkola et al., 2015).The bacterium has been found in the intestines of many animals like domestic pigs, goats, sheep, wild lagomorphs, birds, rodents, and shrews (Niskanen et al., 2003; Laukkanen et al., 2008; Giannitti et al., 2014; Le Guern et al., 2016; Joutsen et al., 2017). It can also thrive in soil as well as in certain protozoans and nematodes (Buzoleva and Somov, 2003; Gengler et al., 2015; Santos-Montañez et al., 2015).

Adaptation to cold and long-term growth at low temperatures poses many challenges to bacteria. Low temperature decreases the fluidity of cell membranes, thereby interfering with normal membrane protein function. It also slows down protein folding, ribosomes, and translation as well as excessively stabilizes nucleic acid structures (Palonen et al., 2010). Cold also induces radical oxygen species production by both slowing metabolism and increasing oxygen solubility (Chattopadhyay et al., 2011). Psychrotrophic bacteria have many methods to deal with these problems. For example, bacteria can increase membrane fluidity by introducing unsaturated lipids that no longer fit as snugly together (Suutari and Laakso, 1994). Unsaturated fatty acids dominate the fatty acid composition of *Y. pseudotuberculosis* at low temperatures (Bakholdina et al., 2004).

Cold shock proteins (Csp) are small proteins whose mRNA carries a cold shock domain that enables its translation at low temperatures. Csps, along with helicases and translation factors exhibiting similar stabilizing secondary structures, unwind mRNA and support the translational apparatus at low temperatures. *Y. pseudotuberculosis* has nine *csp* genes, homologous to those of *Escherichia coli*, five of which are induced at low temperature in *E. coli* (Keto-Timonen et al., 2016). The bacterium also has five helicases with a conserved DEAD-box motif: CsdA (*yptb0486*), RhlE (*yptb1214*), RhlB (*yptb0165*), DbpA (*yptb1652*), and SrmA (*yptb2900*).

The main goal of this study was to determine how *Y. pseudotuberculosis* manages to thrive at refrigerator temperature. We identified which genes showed significantly more transcripts at 3°C when compared to 28°C, at each growth phase, mainly focusing on the beginning of growth and logarithmic phase. Of all cold shock proteins, those encoded by *yptb1423*, *yptb3585*, *yptb3586*, and especially *yptb2414* and *yptb2950*, showed significantly more transcripts at low temperature,

seemingly forming the backbone of cold acclimation of *Y. pseudotuberculosis*. Furthermore, we found that, in addition to CsdA, helicases RhlE, and DbpA were significantly upregulated at low temperature, which speaks to their importance in surviving low temperatures. The bacterium also seems to accumulate glycine betaine by uptake and biosynthesis, as the corresponding genes were upregulated at low temperature. Transcription termination factor Rho, along with IF-1 and RbfA, both acting on ribosomes, were also upregulated, which would seem to suggest that they play an important role. None of these genes and proteins have, to our knowledge, been linked to cold acclimation of *Y. pseudotuberculosis* before.

MATERIALS AND METHODS
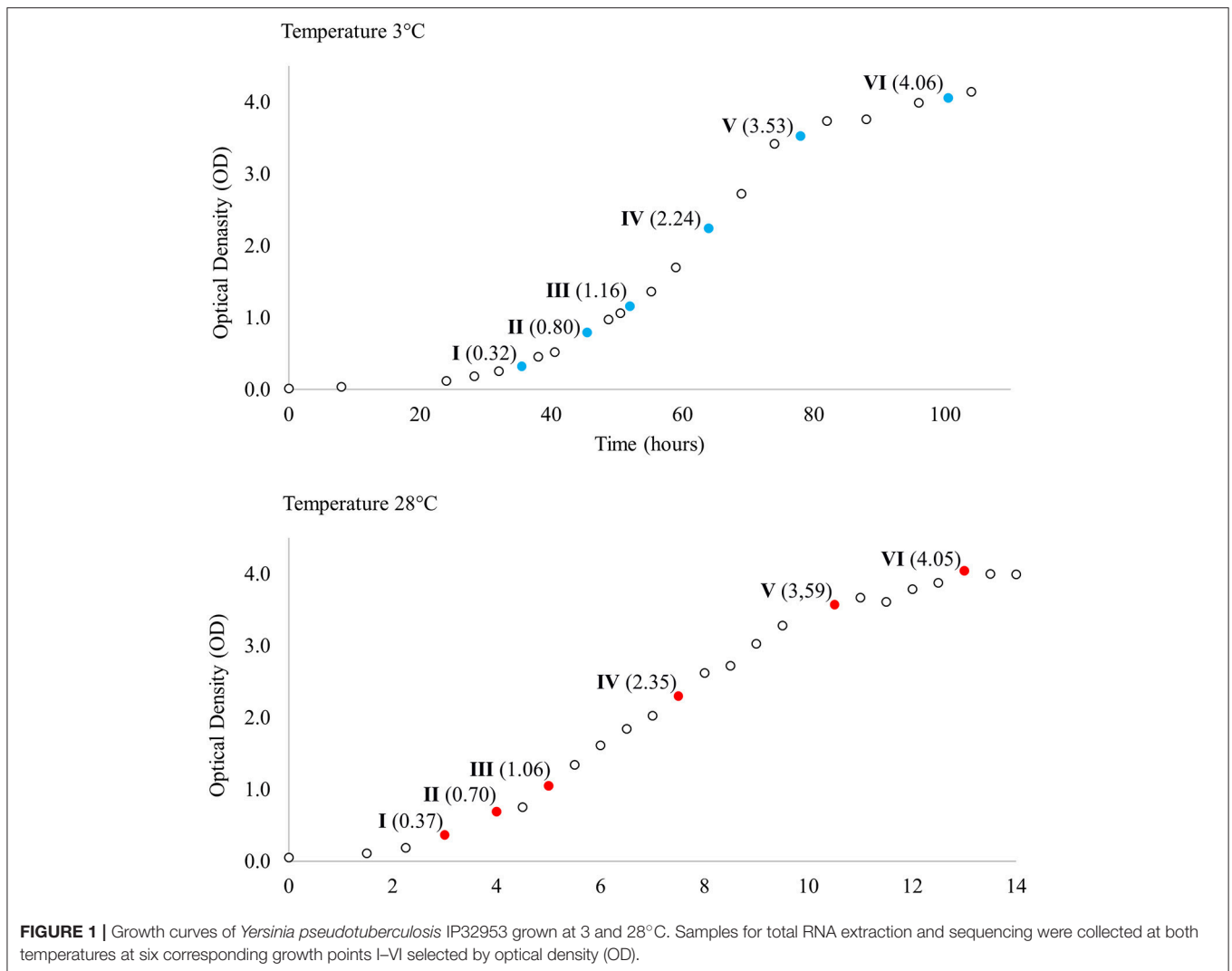
Bacterial Strain and Growth Conditions

Single *Y. pseudotuberculosis* IP32953 colonies grown on blood agar plates at 28°C were inoculated and grown separately in LB broth (Luria-Bertani; Sigma-Aldrich, St. Louis, MO, USA) at 28°C with shaking overnight. Overnight broths were diluted (1:100) in LB broth and divided into two groups so that four cultures (biological replicates) were grown with shaking both at 3°C and at 28°C. Biological replicates were used to better model true biological variability and improve the accuracy of statistical methods. Samples for total RNA extraction were collected at six corresponding points at different phases of growth across both temperatures (**Figure 1**). For total RNA extraction 1.25 ml of bacterial culture was mixed with 250 µl cold phenol-ethanol mixture (1:10) and kept on ice for 30 min. After incubation, samples were centrifuged at 4°C at 13,200 rpm for 2 min and the resulting cell pellets were stored at −70°C until RNA isolation.

RNA Isolation

Total RNA was isolated using GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) and treated with DNA-Free DNA Removal Kit (Ambion, Life Technologies, Carlsbad, CA, USA) according to manufacturers' instructions. The quantity and quality of RNA was examined with a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA was stored at −70°C until RNA-seq library preparation.

Library Preparation and Sequencing

Ribosomal RNA was depleted from total RNA using Ribo-Zero rRNA Removal Kit for Bacteria (Epicenter, Madison, WI, USA) following the manufacturer's protocol. cDNA libraries were prepared using Script-Seq v2 RNA-Seq Library Preparation Kit (Epicenter) following the manufacturer's instructions. The libraries were amplified twice by PCR and barcoded. PCR was performed using Phusion High-Fidelity DNA Polymerase Kit (Thermo Fisher Scientific). The libraries were purified using AMPure XP System (Beckman Coulter, Brea CA, USA) after both PCR procedures. The libraries were sequenced (75 bp single read) using the Illumina NextSeq500 platform at the Institute of Biotechnology, University of Helsinki, yielding four sets of sequence reads (biological replicates) per growth point at both

FIGURE 1 | Growth curves of *Yersinia pseudotuberculosis* IP32953 grown at 3 and 28°C. Samples for total RNA extraction and sequencing were collected at both temperatures at six corresponding growth points I–VI selected by optical density (OD).

3 and 28°C. Raw sequences were deposited in the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number SRP144570.

cDNA Synthesis and RT-qPCR

To confirm the differences of transcript levels identified in the RNA-sequence expression analysis, RT-qPCR validation was performed for selected genes (*betB*, *csdA*, *fabF*, *infA*, *mdtI*, *proX*, *rhlE*, and *rho*) at growth points I and III at both temperatures (3 and 28°C). These genes or operons they represent are discussed in detail in this paper. A total of 500 ng of each RNA sample from three biological replicates per growth point was reverse-transcribed into cDNA in duplicate by using Maxima cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Primers for RT-qPCR were designed using Primer-BLAST software ((Ye et al., 2012); **Table 1**). Two replicate qPCR reactions for each cDNA sample were performed using the Dynamo Flash SYBR Green qPCR Kit (Thermo Scientific). Each reaction consisted of 1x Master Mix, 0,5 μM of forward and reverse primer and 4 μl of 1:20 (gene of interest)

or 1:100,000 (16S *rrn*) diluted cDNA in a total volume of 20 μl. Rotor-Gene Q thermal cycler (Qiagen GmbH, Hilden Germany) was used in PCR runs with the cycling protocol consisting of initial heating step at 95°C for 7 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, extension at 72°C for 20 s, and a final extension at 60°C for 1 min. After each run a melt curve analysis was done to confirm specificity. Amplification reaction efficiencies for each primer pair were obtained from RT-qPCR standard curves prepared from serial dilutions of pooled cDNA samples. The duplicate C_q values for PCR replicates were averaged. The relative quantification of gene of interest transcript levels at 3°C, normalized to reference gene (16S *rrn*) transcript levels and calibrated to the samples taken at the same growth point at 28°C, were calculated using the Pfaffl method (Pfaffl, 2001). Given near equal primer efficiencies, the logFC values of Pfaffl gene expression ratios between 3 and 28°C should be proportional to the logFC values acquired by RNA-Seq. A linear regression analysis was performed on corresponding logFC values of RNA-Seq and RT-qPCR.

TABLE 1 | Primers used in quantitative real-time reverse transcription-PCR.

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
16S rRNA gene	GCTCGTGTTGTGAAATGTTGG	TATGTGGTCCGCTGGCTCT
betB	AGGATTTGAACCGTGCCCAT	GCTGATACCGTTTTCACGGC
csdA	GTGATGTTGGCGAGATGGAG	ATCGTTGAGTGGGAAGCAAA
fabF	ATGCTGATGTCATGGTGGCT	TGCTGCTTGTGGGTTATCGT
infA	TCTTGATACGCTGCCGAACA	TGACTTTGTCACCCGTCAGG
mdtI	CCCGCCAGCAGTAAGATCAA	ATGTGGGGCGGTTTTGGTAT
proX	GATAACCCTGTGCCAAGCCT	GATGCAACCTATCTGGCGGT
rhlE	ATAGCGGCAAGGTGAAACCA	ATCCTGCGTGCTGTTGAAGA
rho	CGTTAGTTTTCGCAGTCGCC	CCGCTCTGGTACCCGTAAAG

Alignment and Annotation of RNA-Seq Data

We aligned, annotated, and analyzed the sequence reads with Bioconductor (Huber et al., 2015). The complete reference genome sequence and genomic features of *Y. pseudotuberculosis* IP32953 (Johnson et al., 2015) were acquired from the Pathosystems Resource Integration Center, PATRIC (Wattam et al., 2014). The sequence reads were both aligned to the reference genome, allowing a maximum of 10 hits, and annotated with Bioconductor package QuasR (Gaidatzis et al., 2015).

Differential Gene Expression Analysis

One of the replicates of growth point IV at 28°C was discarded for its low alignment quality. The remaining annotated transcript counts were analyzed using Bioconductor package baySeq (Hardcastle and Kelly, 2010). Low count reads were filtered out. The replicate counts for each growth point at 3°C were compared to the counts of the respective growth point at 28°C. The prior distributions were acquired using a negative binomial distribution whose parameters were estimated by quasi-maximum-likelihood methods with a sample size of 10,000. The posterior likelihoods were established using 10 iterations to re-estimate the priors. Results were normalized by library size and results with FDR >0.05 were discarded. Log$_2$ fold changes (logFC) across the two temperatures were calculated using transcript count averages of replicates, and genes with logFC ≥ 2 were considered significantly expressed at 3°C. For ease of viewing, genes were further grouped into operons retrieved from ProOpDB (Taboada et al., 2012). Amino acid sequence similarities were derived from multiple sequence alignment by Clustal Omega (Sievers et al., 2011). Upregulated genes sharing similar functions were classified into functional units (modules), acquired from the KEGG database (Kanehisa and Goto, 2000), using Bioconductor package clusterProfiler (Yu et al., 2012). *P*-values were adjusted for multiple comparisons by controlling the false discovery rate, using the Benjamini & Hochberg method implemented in clusterProfiler (Benjamini and Hochberg, 1995).

Clustering

Expression profiles of genes that were significantly more expressed at low temperature, at least at one growth point, were clustered using Pearson correlation distance. Count data was normalized for visualization by using median ratio normalization implemented in Bioconductor package DESeq2 (Love et al., 2014). Averages of replicate counts were used instead of individual counts. Normalized data for each gene (rows) across all growth points (columns) was then plotted in a heatmap (**Figure 2**).

RESULTS

Expression profiles of *Y. pseudotuberculosis* IP32953 grown at 3 and 28°C were compared at corresponding phases of the growth based on growth curves determined by the optical density. We found 570 genes in total that showed significantly more transcripts at 3°C than 28°C at least at one of the growth points I–VI (**Table 2**). Motility and chemotaxis genes were at the top throughout growth since *Y. pseudotuberculosis* is non-motile at 28°C. The total number of upregulated genes was 482 when motility, chemotaxis, and tRNA genes were filtered out. Growth point VI held the fewest significantly expressed genes ($N = 125$) whereas growth points II and IV held the most ($N = 162$; **Table 2**). The difference in expression was largest for genes involved in motility, with a maximum logFC of 7.42.
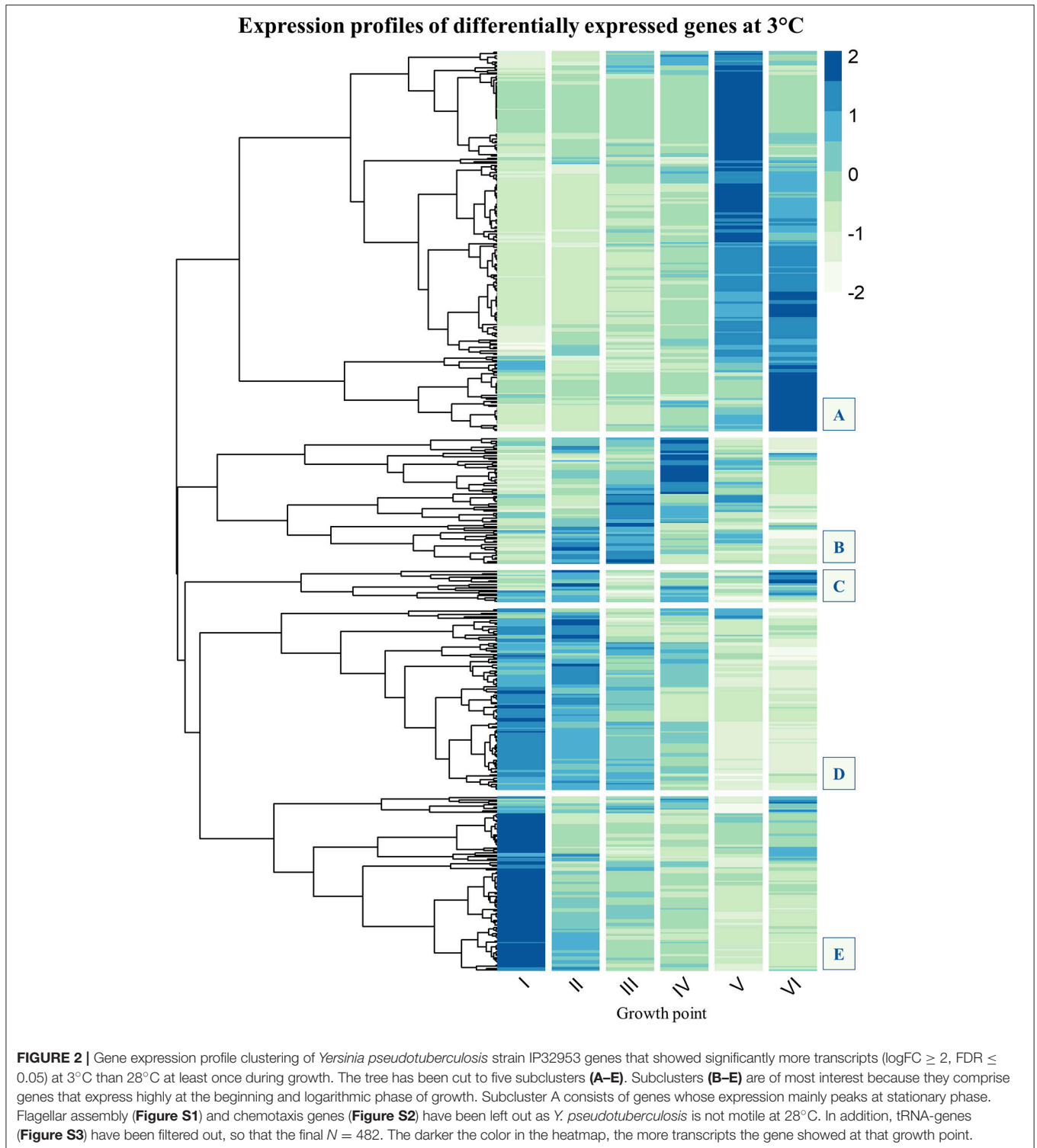
A gene that was expressed both differentially and in great numbers at 3°C probably plays an important role in cold growth. We clustered expression profiles of genes that at least once during growth showed more transcripts at 3°C than at 28°C. Five subclusters (A–E) could be identified in the expression profile heatmap (**Figure 2**). The genes of most interest in the context of cold growth express highly at the beginning and logarithmic phases of growth, clustered into subclusters B–E (see details in **Supplementary Material**). Subcluster A mostly held genes that were expressed highly at the stationary phase.

Validation of RNA Sequencing Results With RT-qPCR

The expression profiles of eight differentially expressed genes at two different growth points were evaluated by RT-qPCR and compared with the RNA-seq analysis results. In linear regression analysis between the RNA-seq and RT-qPCR logFC values (**Figure 3**), a 0.93 Pearson correlation coefficient value ($R^2 = 0.85$) was observed, which confirmed the reproducibility and reliability of the RNA-seq method (**Figure 3**).

Differentially Expressed Genes by Growth Point

At growth point I, genes involved in acquiring compatible solutes and various nutrients showed significantly more transcripts at 3°C, and this pattern continued until stationary phase (**Figure 4**). For example, a significant increase in transcripts at 3°C was displayed in the following genes: genes encoding a glycine betaine transporter (*yptb2959–61*); phosphotransferase systems (PTS) to import fructose (*yptb1329–31*; **Figure 5**), N-acetylglucosamine (*yptb1120*, *yptb3075–82*), and L-ascorbate (*yptb2600–2*; **Figure 5**); and ATP-binding cassette (ABC) transporters of maltose (*yptb2521*, *yptb3095–102*) and

FIGURE 2 | Gene expression profile clustering of *Yersinia pseudotuberculosis* strain IP32953 genes that showed significantly more transcripts (logFC $\geq$ 2, FDR $\leq$ 0.05) at 3°C than 28°C at least once during growth. The tree has been cut to five subclusters **(A–E)**. Subclusters **(B–E)** are of most interest because they comprise genes that express highly at the beginning and logarithmic phase of growth. Subcluster A consists of genes whose expression mainly peaks at stationary phase. Flagellar assembly (**Figure S1**) and chemotaxis genes (**Figure S2**) have been left out as *Y. pseudotuberculosis* is not motile at 28°C. In addition, tRNA-genes (**Figure S3**) have been filtered out, so that the final $N = 482$. The darker the color in the heatmap, the more transcripts the gene showed at that growth point.

aldopentoses (*yptb3591–6*). A significant portion of upregulated genes at this growth point were associated with different PTSs and glycine betaine transport (**Figure 4**). As expected, genes encoding chaperone molecules such as helicase RhlE (*yptb1214*) and Csps (*yptb2950, yptb2414, yptb3585,* and *yptb3586*), which

destabilize nucleic acid secondary structures, showed more transcripts at 3°C.

At growth point II, genes involved in further processing of compatible solutes, spermidine efflux, synthesizing desaturated membrane lipids, biosynthesis of ribosomes, securing translation

TABLE 2 | The number of *Yersinia pseudotuberculosis* strain IP32953 genes expressed significantly more at 3°C than at 28°C by growth point.

Growth point (OD)	logFC	
	≥2	≥4
I (0.32–0.37)	134	27
II (0.69–0.80)	162	13
III (1.05–1.17)	139	9
IV (2.24–2.30)	162	9
V (3.53–3.57)	129	7
VI (4.05–4.06)	125	8

Flagellar assembly and chemotaxis genes have been disregarded as Y. pseudotuberculosis is not motile at 28°C; and tRNA-genes have also been filtered out.

under cold stress, and posttranscriptional modification of RNA molecules showed significantly more transcripts at 3°C. Modules of different amino acid transporters and betaine synthesis were overexpressed (**Figure 4**). Genes encoding translation factors IF-1 (*yptb1395*) and Rho factor (*yptb0167*) peaked at this growth point. New additions to differentially expressed chaperone genes were helicase gene *dbpA* (*yptb1652*) and a new Csp gene (yptb1423).

At growth point III, a urease operon (*yptb2938–44*) and PTSs to import fructose (**Figure 5**), N-acetylglucosamine, and mannose showed significantly more transcripts at 3°C than 28°C. The gene encoding superoxide dismutase (*yptb3925*) also showed more transcripts at 3°C at this growth point. At growth point IV, it seems the nutrient strategy shifted as an operon involved in sulfur metabolism (*yptb2309–14*) was expressed more at 3°C than 28°C. In addition to genes encoding IF-1 and Rho factor, a new translation factor gene *rbfA* (*yptb0481*) showed more transcripts at 3°C at this point of growth.

At growth points V and VI, the nutrient strategy continued shifting as operons involved in the metabolism of histidine (*yptb1965–9*, *yptb3851–2*; **Figure 5**), cystine (*yptb1717–20*; **Figure 5**), methionine (*yptb2973–5*; **Figure 5**), and nitrogen compounds (*yptb0022–3*) showed more transcripts at 3°C. At the same time, Csp gene expression levels and differences tapered off. The gene encoding helicase CsdA (*yptb0486*) was significantly more expressed at 3°C at growth point III, but at 28°C at growth points V and VI. An operon encoding a system that utilizes autoinducer AI-2 showed significantly more transcripts at growth point V.

Expression of Cold Shock Protein Genes

Of all the Csp genes, *yptb2414* showed most transcripts at 3°C from the beginning of growth throughout the logarithmic growth phase (**Figure 6**). Its difference in expression between 3 and 28°C was significant at all growth points and the largest of all Csp genes. Gene *yptb1624* was expressed significantly more at 28°C (**Figure 6**). Gene *yptb2950* showed many more transcripts at 3°C at growth points I–IV, with a peak logFC of over 7 (**Figure 6**). Its transcription counts dipped after growth point I and decreased toward stationary phase. Gene *yptb1423* was expressed at lower levels, but showed significantly more transcripts at 3°C throughout growth points II–IV (**Figure 6**).
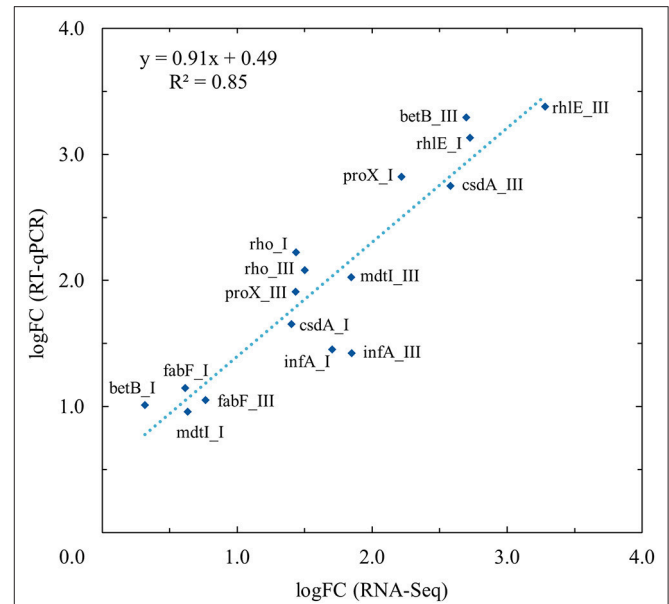

FIGURE 3 | Validation of RNA-Seq results with quantitative real-time reverse-transcription PCR (RT-qPCR) using linear regression analysis. RT-qPCR validation was performed for selected genes (*betB*, *csdA*, *fabF*, *infA*, *mdtI*, *proX*, *rhlE*, and *rho*) at growth points I and III at both temperatures (3 and 28°C). Linear regression analysis showed an R^2 coefficient of determination value of 0.85 between the RNA-Seq and RT-qPCR log₂ fold changes (logFC).
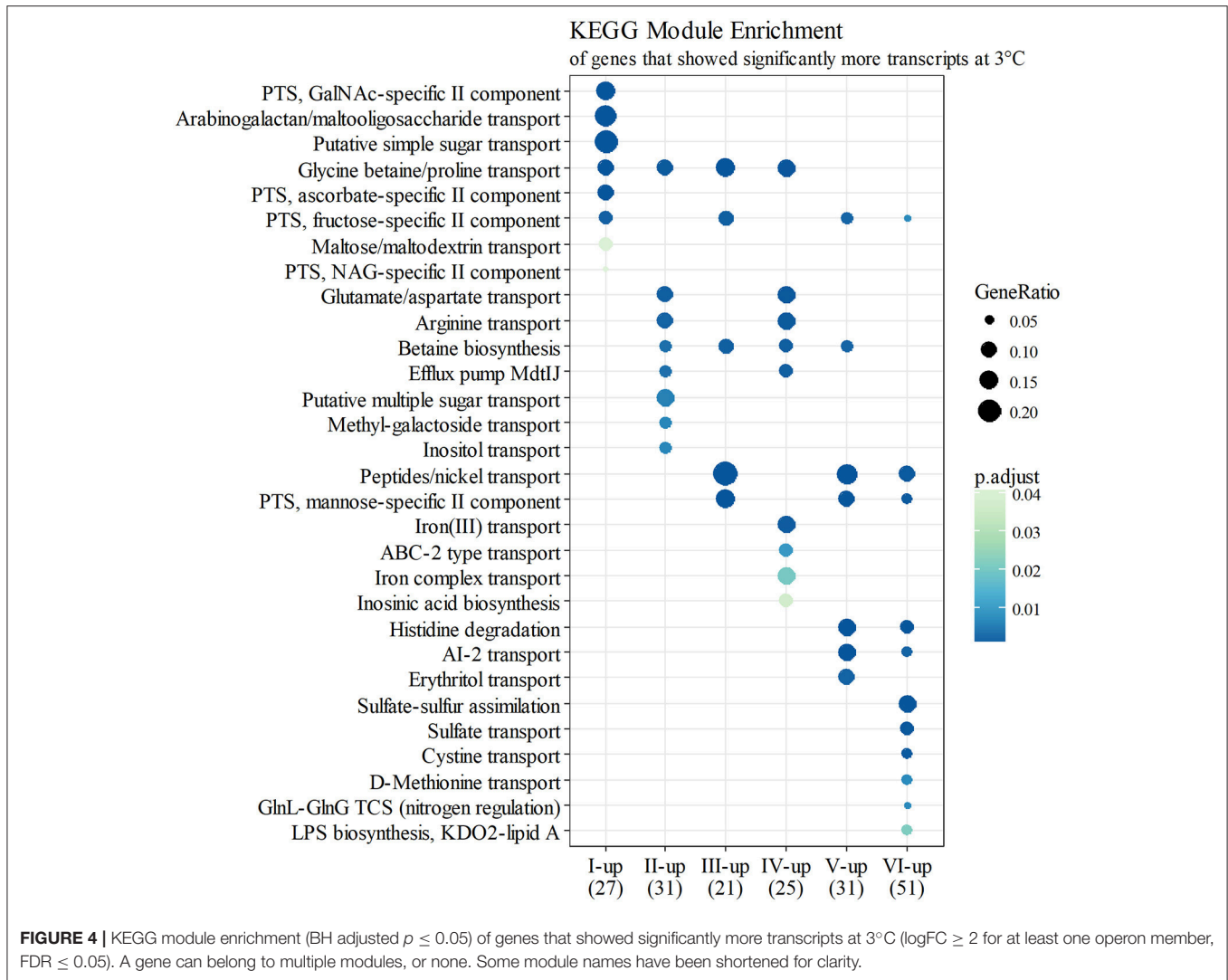
Nearly identical genes, *yptb3585* and *yptb3586*, were expressed highly at growth point I, but their expression levels dipped right after (**Figure 7**). However, the differences in their expression between 3 and 28°C consistently favored cold at significant levels. Expression levels of *yptb3587* and *yptb1088* rose toward the stationary phase at 3°C, but the difference between 3 and 28°C was not significant. After starting growth, *yptb3587* appeared slightly downregulated (**Figure 7**). Gene *yptb1392* was expressed much in the same way, but showed significantly more transcripts at 28°C at the stationary phase of growth (**Figure 7**).

Expression of DEAD-Box RNA Helicase Genes

Y. pseudotuberculosis has five helicases with a conserved DEAD-box motif: CsdA (*yptb0486*), RhlE (*yptb1214*), RhlB (*yptb0165*), DbpA (*yptb1652*), and SrmA (*yptb2900*). All the helicase genes were expressed more at 3°C throughout growth (**Figure 8**), but the difference was significant for *csdA* (*yptb0486*) at growth point III, *rhlE* (*yptb1214*) at growth points I–IV, and *dbpA* (*yptb1652*) at growth points II and IV.

Expression of Genes Handling Compatible Solutes

Genes *yptb2959–61* form the *proU* operon that encodes a transport system for glycine betaine and proline; genes *yptb1195–98* form the *betIBA-betT* divergent operon that is involved in glycine betaine biosynthesis; and genes *yptb2052* and *yptb2051* form the *mdtIJ* operon that encodes a spermidine efflux

FIGURE 4 | KEGG module enrichment (BH adjusted $p \leq 0.05$) of genes that showed significantly more transcripts at 3°C (logFC ≥ 2 for at least one operon member, FDR ≤ 0.05). A gene can belong to multiple modules, or none. Some module names have been shortened for clarity.

pump. Operon *proU* showed significantly more transcripts at 3°C at growth points I–IV (**Figure 9**), operon *betIBA-betT* at growth points II–IV (**Figure 10**), and operon *mdtIJ* at II and IV (**Figure 11**). Corresponding functional modules were also overexpressed at growth points I–IV for operon *proU*, points II–V for operon *betIBA-betT*, and points II and IV for operon *mdtIJ* (**Figure 4**). Another glycine betaine and L-proline transporter encoding gene *proP* (*yptb0608*) showed more transcripts at 3°C throughout the growth, but not quite at significant levels (**Figure 9**).

Expression of Genes Encoding Rho Factor, IF-1, and RbfA

Genes *rho* (*yptb0167*), *infA* (*yptb1395*), and *rbfA* (*yptb0481*) encode the homologs of Rho factor, the translation initiation factor IF-1, and ribosome binding factor RbfA, respectively. The three factor genes were expressed highly from the beginning of growth to the end of the logarithmic phase (**Figure 12**). Their difference in expression between 3 and 28°C was significant at

growth points II and IV, over 16-fold at the largest for *yptb1395*, but the three genes showed more transcripts at 3°C consistently throughout the growth.

Expression of Genes Involved in Modifying Membrane Lipid Composition

Genes *yptb1450* and *yptb2469*, encoding FabA and FabF, were expressed highly at the beginning of growth whereas gene *yptb2426* encoding FabB at stationary phase (**Figure 13**). Gene *yptb2469* showed significantly more transcripts at 3°C at growth point II, although the difference favored cold during most of the growth.

DISCUSSION

We identified several upregulated genes that may be important to cold adaptation of *Y. pseudotuberculosis*. We also analyzed gene expression profiles to better understand what happens at different phases of growth in cold. To tackle problems
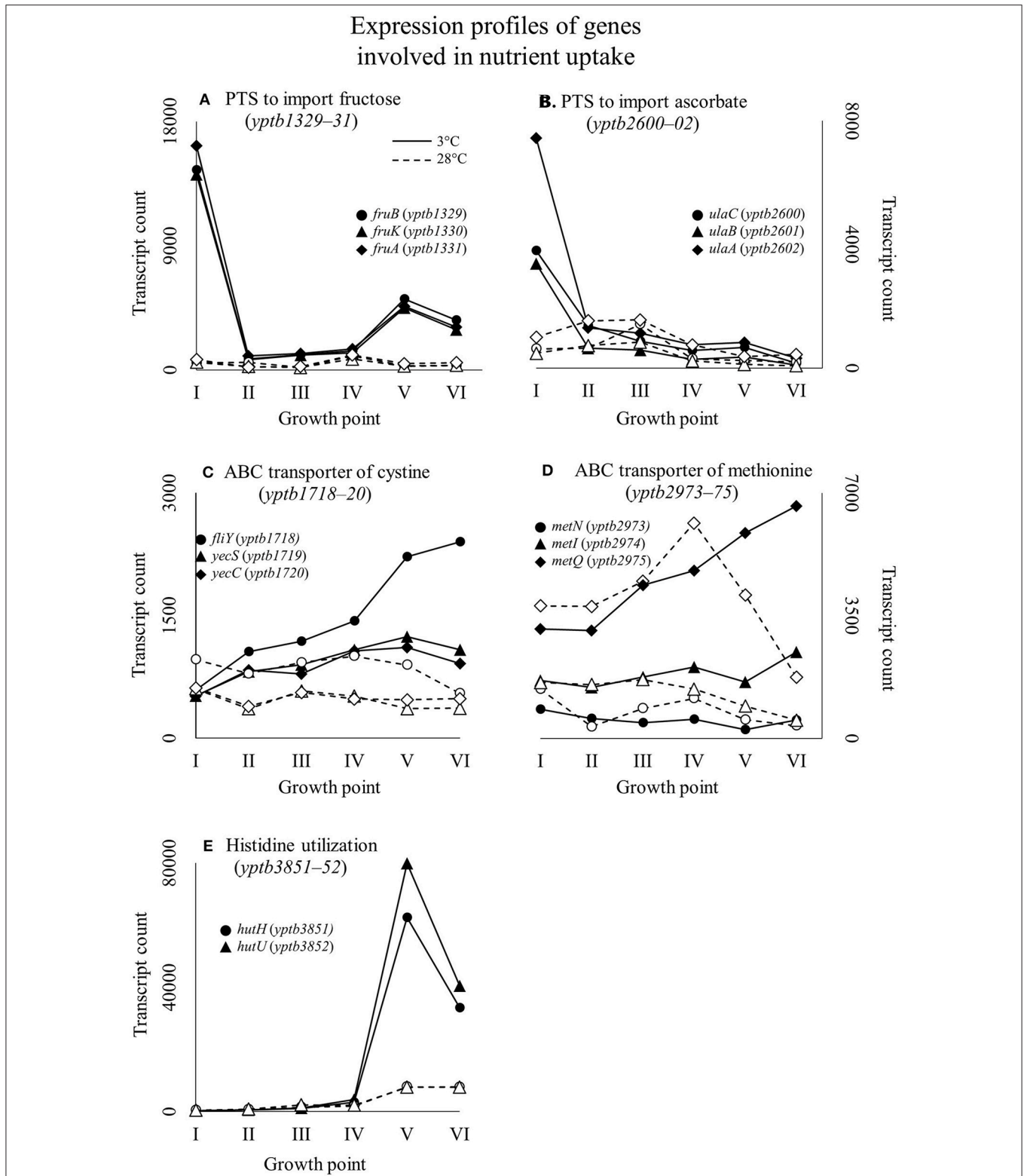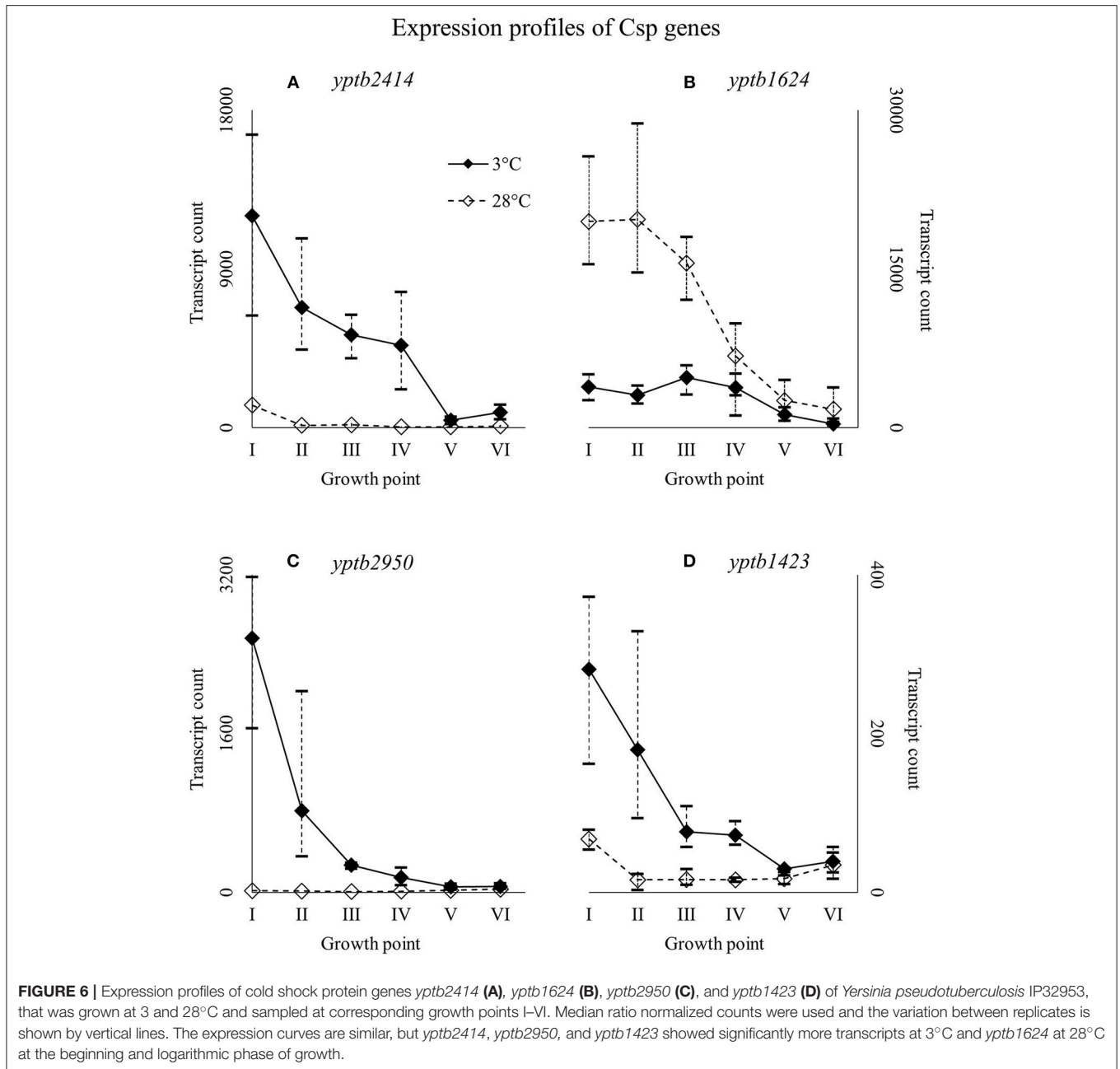
FIGURE 5 | Expression profiles of example genes involved in nutrient uptake: *fru*-operon encoding a phosphotransferase system (PTS) to import fructose **(A)** ; *ula*-operon encoding a PTS to import ascorbate **(B)**; ATP binding cassette (ABC) transporter of cystine, dimer form of cysteine **(C)**; ABC transporter of methionine **(D)**; and part of the histidine utilization system **(E)** of *Yersinia pseudotuberculosis* IP32953, that was grown at 3 and 28°C and sampled at corresponding growth points I–VI. Median ratio normalized counts were used. A large significant difference in expression of sugar importers between 3 and 28°C can be observed at the beginning of growth. Toward the end of growth at 3°C, amino acid utilization takes precedence when sugar importer expression tapers off.

FIGURE 6 | Expression profiles of cold shock protein genes *yptb2414* **(A)**, *yptb1624* **(B)**, *yptb2950* **(C)**, and *yptb1423* **(D)** of *Yersinia pseudotuberculosis* IP32953, that was grown at 3 and 28°C and sampled at corresponding growth points I–VI. Median ratio normalized counts were used and the variation between replicates is shown by vertical lines. The expression curves are similar, but *yptb2414*, *yptb2950,* and *yptb1423* showed significantly more transcripts at 3°C and *yptb1624* at 28°C at the beginning and logarithmic phase of growth.

posed by refrigerator temperatures, *Y. pseudotuberculosis* has an extensive toolbox that includes Csps, DEAD-box RNA-helicases, compatible solutes, transcription factors, and fatty acid saturases.

Nutrient Acquisition

Y. pseudotuberculosis expresses many genes involved in securing nutrients significantly more at 3°C compared to 28°C (**Figures 4,5**), as successful growth in low temperatures requires more energy. In our results, a shift in nutrient utilization over time is observed. At the beginning of growth, transporters of fructose, ascorbate, maltooligosaccharides,

and maltose were upregulated at 3°C. At the beginning of logarithmic phase, transporters of glutamate, aspartate, and arginine were upregulated. Toward the stationary phase, in addition to an operon turning histidine to glutamate, transporters of cystine, methionine, and sulfates were upregulated.

PTSs allow quick acquisition of carbohydrates from the environment (Postma et al., 1993). PTSs have also been linked to regulatory functions during cold stress (Wouters et al., 2000; Monedero et al., 2007). *Y. pseudotuberculosis* expresses several PTSs especially at the beginning of growth significantly more at 3°C than 28°C, as it is in dire need of carbon to sustain
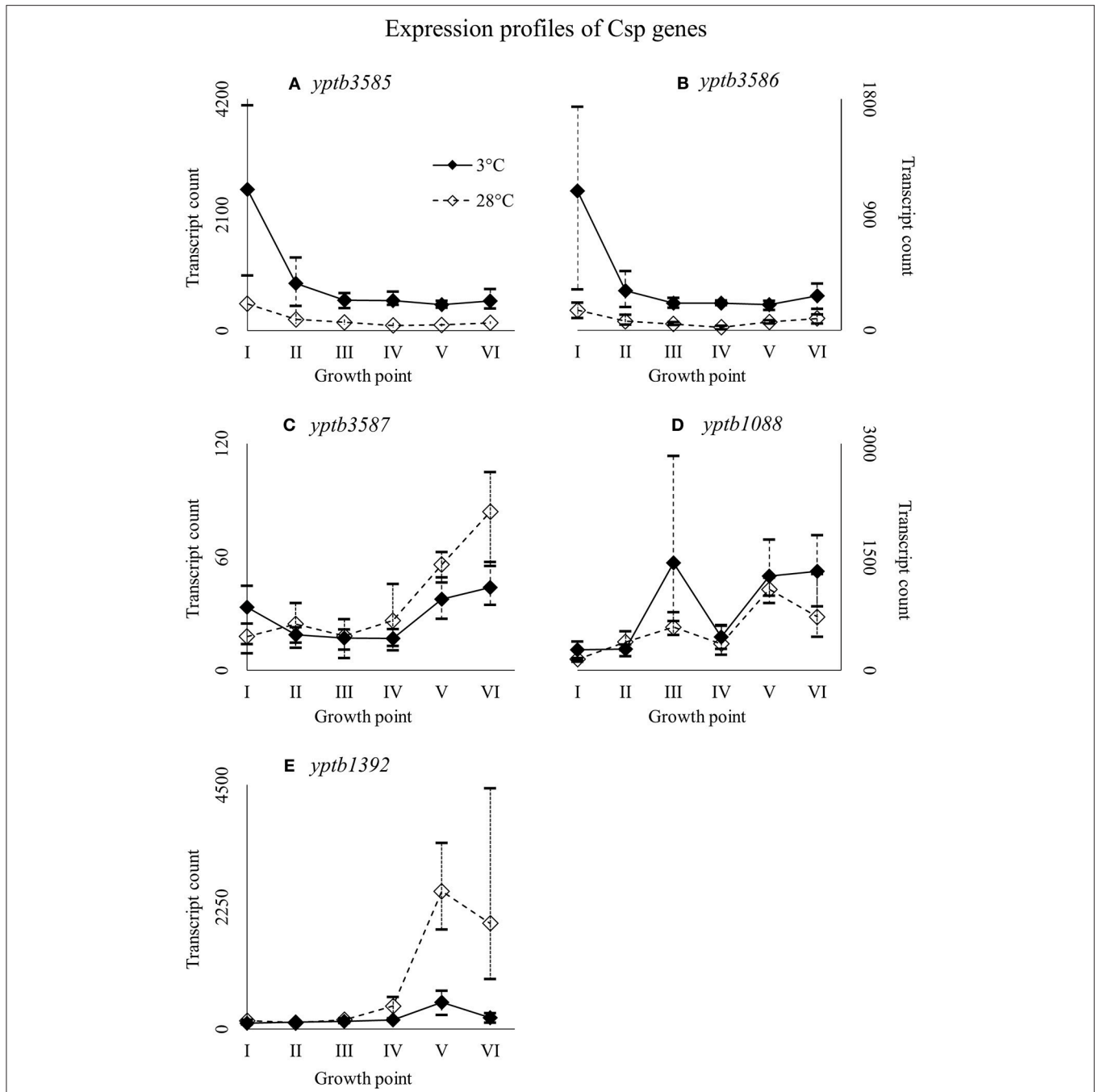
FIGURE 7 | Expression profiles of cold shock protein genes *yptb3585* **(A)**, *yptb3586* **(B)**, *yptb3587* **(C)**, *yptb1088* **(D)**, and *yptb1392* **(E)** of *Yersinia pseudotuberculosis* IP32953, that was grown at 3 and 28°C and sampled at corresponding growth points I–VI. Median ratio normalized counts were used and the variation between replicates is shown by vertical lines. Expression of *yptb3585–86* decreased toward stationary phase whereas that of *yptb3687, yptb1088,* and *yptb1392* increased.

its laborious growth. Another large peak in expression of a fructose PTS can be observed at growth point V at 3°C, whereas the peak is located at growth point IV at 28°C. However, this is probably due to slower regulatory processes at 3°C. In *Y. pestis*, the *malMBKEFG*-operon, which is involved in maltose intake, was highly upregulated after cold shock (Han et al., 2005).

Maltose can act as an cryoprotectant in addition to being a carbon source (Jain and Roy, 2009). In our results, parts of the *mal*-operon were upregulated especially at the beginning of growth.

E. coli has been shown to accumulate certain amino acids, including aspartic acid, glutamic acid, and methionine,
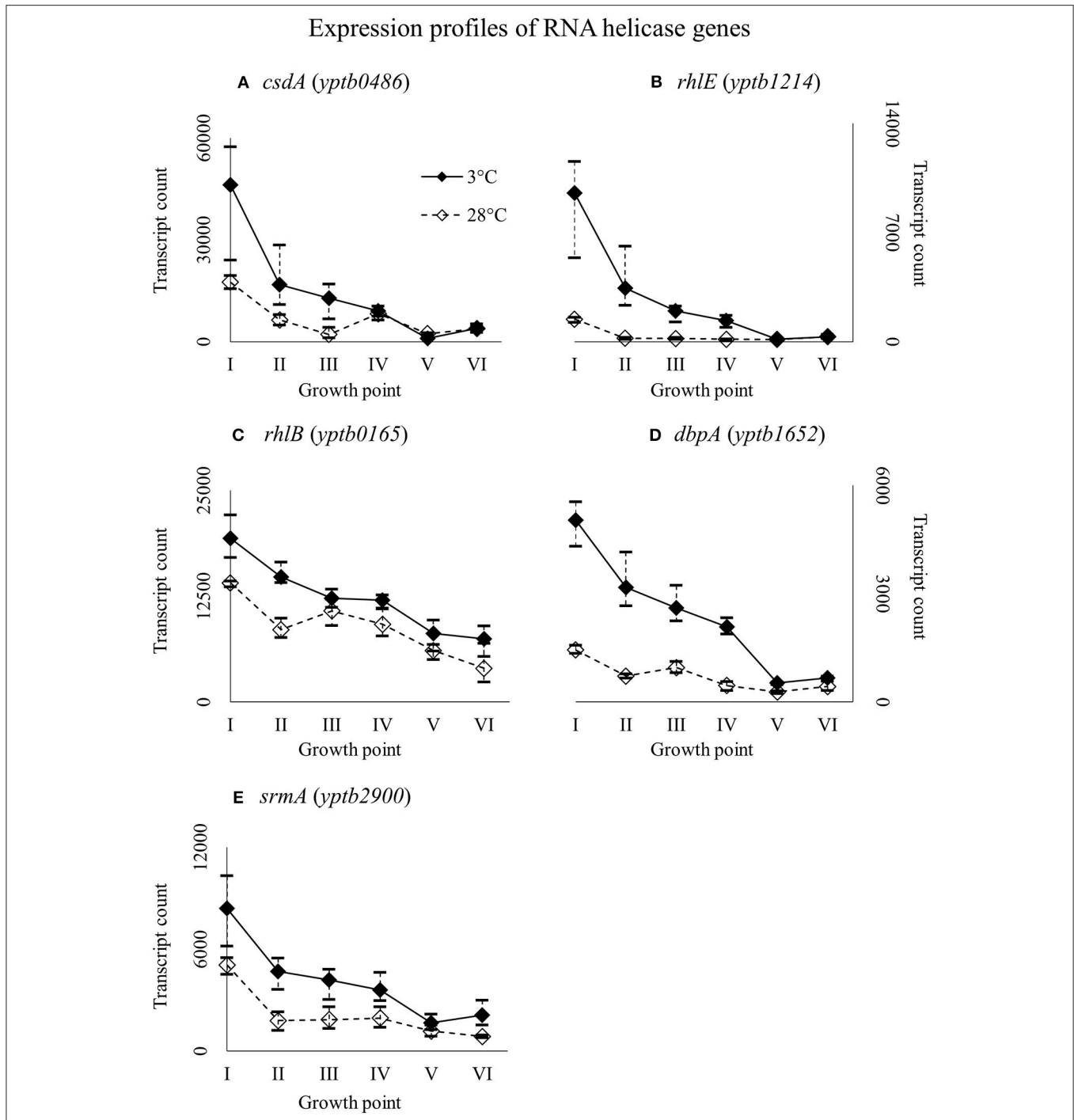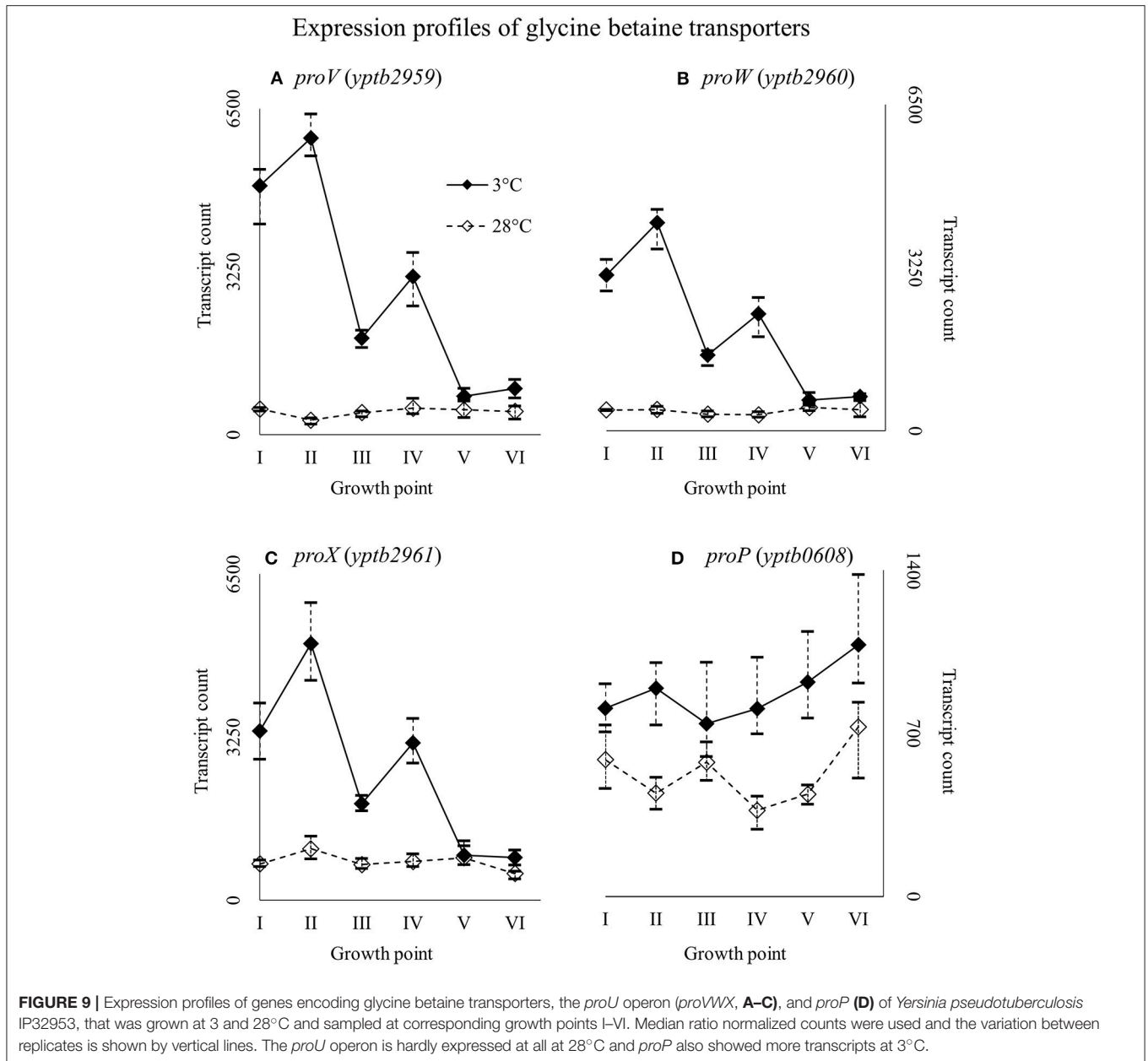
FIGURE 8 | Expression profiles of DEAD-box RNA helicase genes *csdA* **(A)**, *rhlE* **(B)**, *rhlB* **(C)**, *dbpA* **(D)**, and *srmA* **(E)** of *Yersinia pseudotuberculosis* IP32953, that was grown at 3 and 28°C and sampled at corresponding growth points I–VI. Median ratio normalized counts were used and the variation between replicates is shown by vertical lines. Gene *csdA* showed significantly more transcripts at growth point III, *rhlE* at growth points I–IV, and *dbpA* at growth points II and IV, although all the genes expressed more at 3°C at least from the beginning through logarithmic phase.

when subjected to suboptimal temperatures (Jozefczuk et al., 2010). Accumulation has been theorized to result from protein degradation. In our results, ABC transporters for cystine and methionine were upregulated in cold at the stationary phase.

Cold Shock Proteins

Y. pseudotuberculosis seems to express Csps encoded by *yptb2414* and *yptb2950* almost exclusively at low temperature, i.e., with the highest significant difference of all Csps, and at high
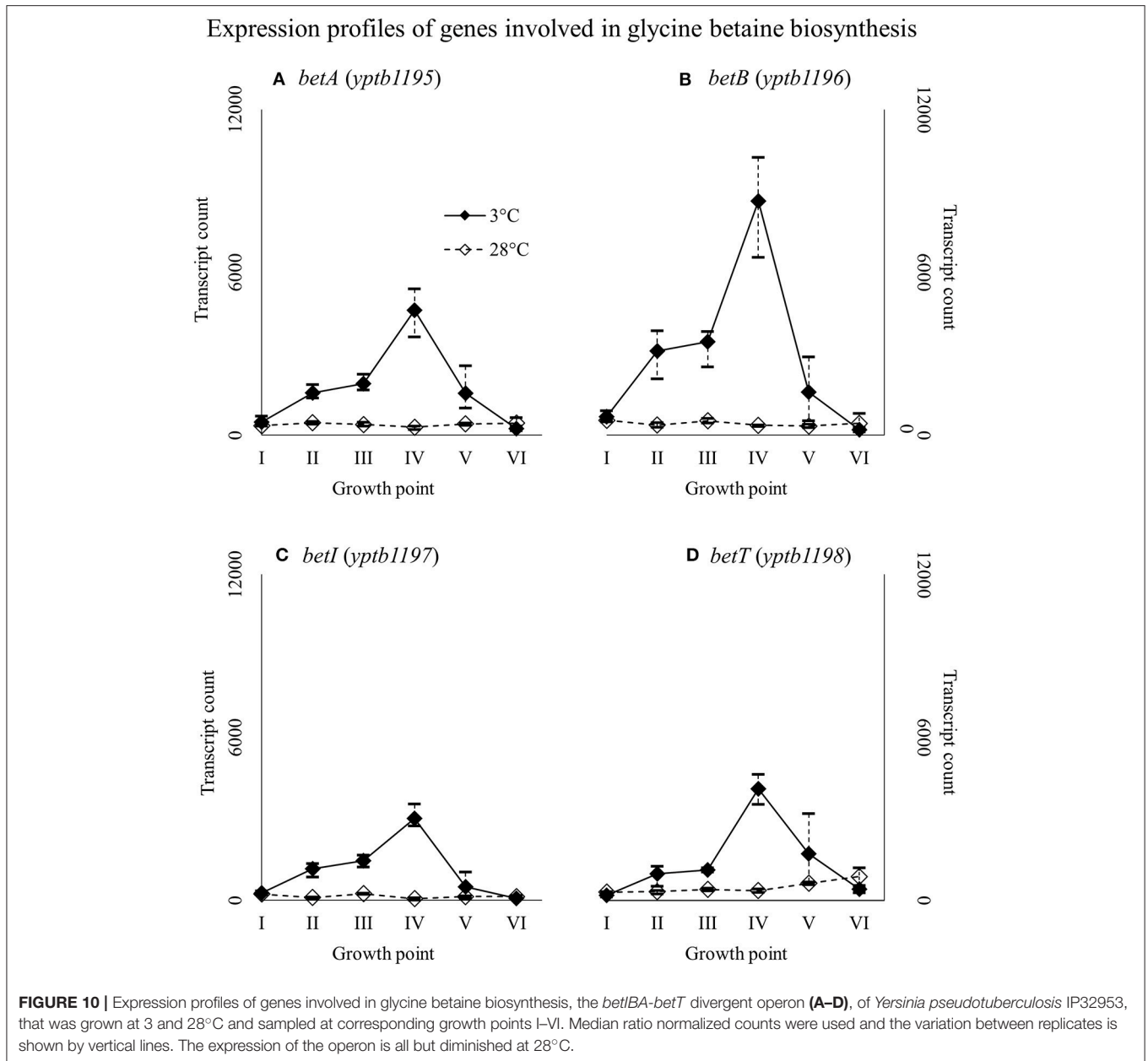
FIGURE 9 | Expression profiles of genes encoding glycine betaine transporters, the *proU* operon (*proVWX*, **A–C**), and *proP* **(D)** of *Yersinia pseudotuberculosis* IP32953, that was grown at 3 and 28°C and sampled at corresponding growth points I–VI. Median ratio normalized counts were used and the variation between replicates is shown by vertical lines. The *proU* operon is hardly expressed at all at 28°C and *proP* also showed more transcripts at 3°C.

levels. Additionally, *yptb3585*, *yptb3586,* and *yptb1423* were upregulated throughout the growth. Csps encoded by *yptb2950* and *yptb3585/6* are comparable to CspG and CspB of *E. coli*, respectively (Keto-Timonen et al., 2016). Csp encoded by *yptb2414* is 80% similar to CspC and CspE of *E. coli* K-12 W3110.

Csp encoded by *yptb1088*, which is almost identical to CspE of *E. coli* (Keto-Timonen et al., 2016), showed more transcripts at 3°C, but not at significant levels. However, its transcript count rose toward stationary phase like has been shown to happen in *E. coli* (Czapski and Trun, 2014). In *E. coli*, CspE was also the most abundant of all *csps* after cold shock and present in the bacterium at all times. The expression of *yptb1392*,
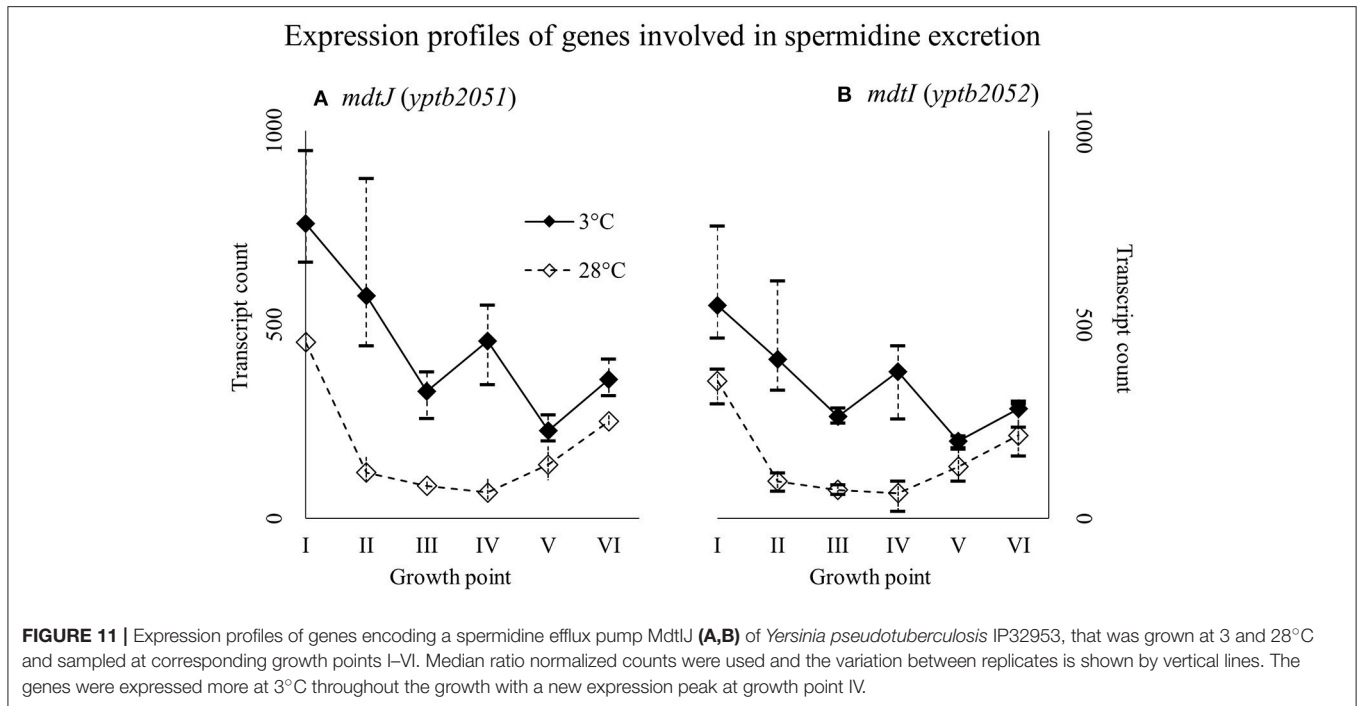
similar to CspD of *E. coli*, ramped up at stationary phase in 28°C and a similar but less dramatic bump can be seen in expression profile in 3°C. In *E. coli*, CspD is not cold-induced and is expressed mainly at stationary phase (Yamanaka et al., 2001). It is involved in persister cell and biofilm formation (Kim and Wood, 2010).

In *E. coli*, the genes *cspA*, *cspB*, *cspE*, and *cspG* all must be deleted to achieve a cold-sensitive phenotype, and the deletion of only one or two genes leads to overexpression of the rest (Xia et al., 2001). In addition, CspC and CspE seem to double as regulatory elements in stress responses (Phadtare and Inouye, 2001; Phadtare et al., 2006). The two proteins have been shown to be required for expression of fructose

FIGURE 10 | Expression profiles of genes involved in glycine betaine biosynthesis, the *betIBA-betT* divergent operon **(A–D)**, of *Yersinia pseudotuberculosis* IP32953, that was grown at 3 and 28°C and sampled at corresponding growth points I–VI. Median ratio normalized counts were used and the variation between replicates is shown by vertical lines. The expression of the operon is all but diminished at 28°C.

PTS at suboptimal temperatures (Phadtare et al., 2006). *E. coli* expresses *cspA, cspB*, and *cspG* even when its protein synthesis is inhibited by antimicrobials (Etchegaray and Inouye, 1999). In *Clostridium botulinum*, deletion of *cspB*, or *cspC* leads to a cold-sensitive phenotype, and *cspC* deletion even hinders growth at 37°C (Söderholm et al., 2011). Genes *cspB* and *cspC* also provide the bacterium resistance to NaCl, pH, and ethanol stress (Derman et al., 2015). In *Staphylococcus aureus*, *cspB* mutation renders the bacterium susceptible to cold and various antimicrobials (Duval et al., 2010). By contrast, CspC has been shown to induce more strongly in response to antimicrobials, hydrogen peroxide, and arsenate than cold (Chanda et al., 2009).

In our experiment, the expression of the genes *yptb3585–86*, comparable to *cspB* of *E. coli* (Keto-Timonen et al., 2016), dipped after the beginning of growth at 3°C but showed more transcripts at 3°C throughout the growth. Pathogenic *Yersinia* have been shown to hold a tandem gene duplication *cspA1/A2* (Neuhaus et al., 1999), which is very nearly identical to genes *yptb3585–87* and needs to be downregulated after the initial cold shock for growth to continue (Neuhaus et al., 2000). In *Yersinia enterocolitica*, *cspA* and *cspB* were the very first genes expressed after cold-shock with their transcript levels decreasing after the beginning (Bresolin et al., 2006). Similarly, *cspA* and *cspB* expression fluctuated accordingly with temperature cycling in *E. coli* (Ivancic et al., 2013). In light of our

FIGURE 11 | Expression profiles of genes encoding a spermidine efflux pump MdtIJ **(A,B)** of *Yersinia pseudotuberculosis* IP32953, that was grown at 3 and 28°C and sampled at corresponding growth points I–VI. Median ratio normalized counts were used and the variation between replicates is shown by vertical lines. The genes were expressed more at 3°C throughout the growth with a new expression peak at growth point IV.

results, *yptb3585–86* might play an important role in the initial regulation of cold-inducible genes also in *Y. pseudotuberculosis*. Genes *yptb2414* and *yptb2950* seem to be important at low temperature growth considering their differential expression and transcript levels. It is also probable that most Csps of *Y. pseudotuberculosis* are interchangeable. Csp encoded by *yptb1423* is an odd one out, with a similarity of 54% at best to other Csps of *Y. pseudotuberculosis* (Keto-Timonen et al., 2016), with a peak similarity of 50% to the Csps of *E. coli* K-12 W3110.

DEAD-Box RNA Helicases

Y. pseudotuberculosis is unable to grow at 3°C without a functional RNA helicase gene *csdA* (*yptb0486*) (Palonen et al., 2012). Of other foodborne pathogens, *Listeria monocytogenes* and *C. botulinum* have been shown to require RNA-helicases, including a CsdA homolog, to grow at suboptimal temperatures (Markkula et al., 2012; Söderholm et al., 2015). In fact, CsdA was the most abundant protein extracted from the microbial mats of Lake Joyce in Antarctica (Koo et al., 2016). CsdA and SrmB of *E. coli* are directly involved in the biogenesis of large ribosomal subunits, presumably at subsequent steps with some functional overlap, and deletion of either gene stunts growth at low temperatures (Charollais et al., 2003, 2004). The deletion of *dbpA* in *E. coli* did not lead to similar accumulation of ribosome precursors and stunted growth at either 25 or 37°C (Peil et al., 2008).
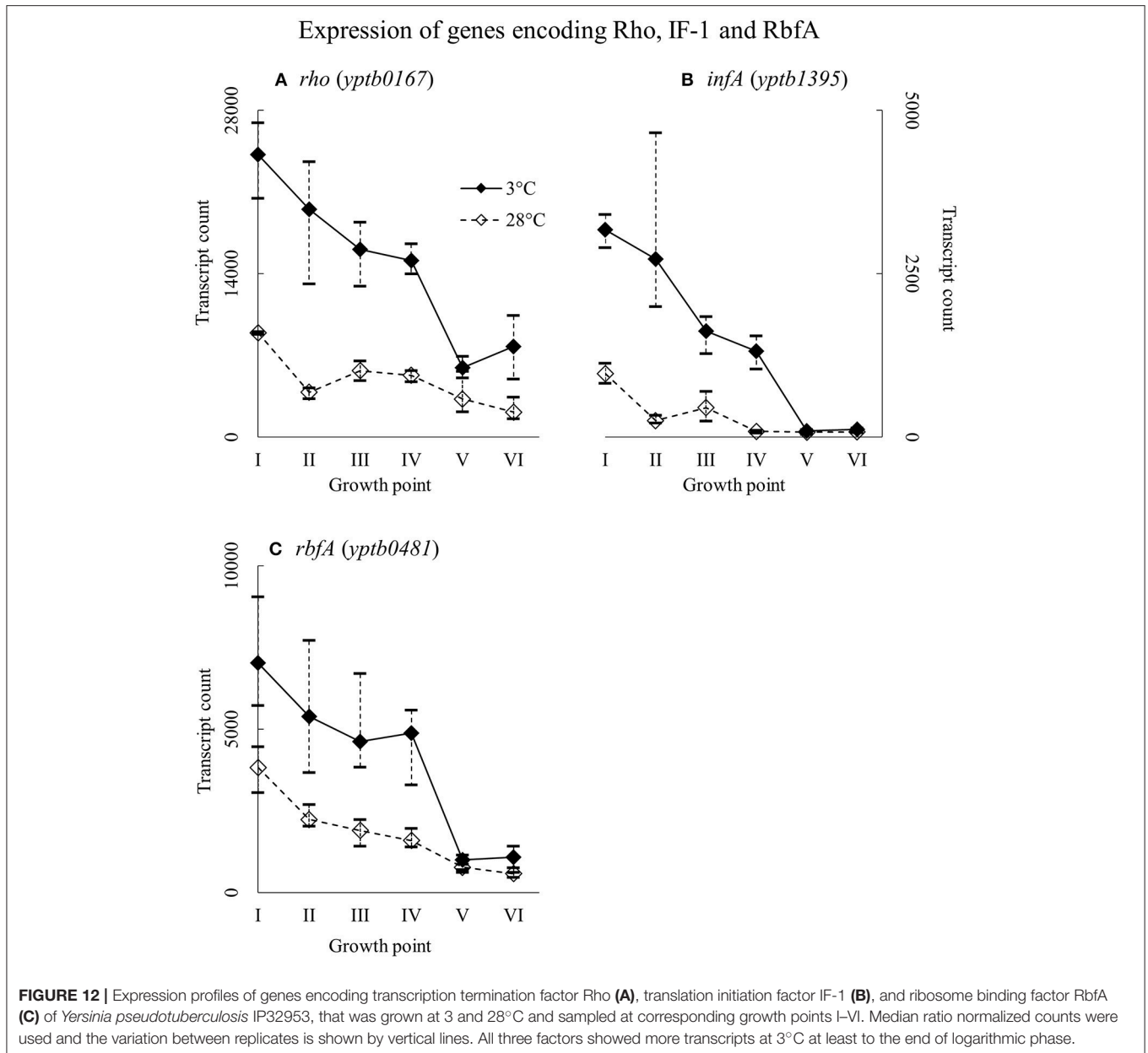
Our results suggest that DbpA (encoded by *yptb1652*) may indeed be important at low temperatures, at least in *Y. pseudotuberculosis*. DbpA is missing completely from *Pasteurellales*, an order of symbionts and parasites growing at unchanging, high-temperature, nutrient-rich niches, which

suggests that DbpA, like other DEAD-box helicases, has become specialized to operate in adverse growth conditions (Iost and Dreyfus, 2006). Amino acid similarities between corresponding helicases of *Y. pseudotuberculosis* IP32953 and *E. coli* K-12 W3110 range from 69 to 90%.

Gene *yptb1214* coding RhlE had the largest and most consistent significant difference in expression in favor of 3°C. It has been suggested by Jain (2008) that RhlE may modulate the function of CsdA and SrmB in priming immature ribosomal RNA. This would also help explain the gene's prominence at low temperature in our results at the beginning and during logarithmic phase when ribosomal activity is at its highest. In *E. coli,* RhlE has been shown to complement the function of both CsdA and RhlB (Khemici et al., 2004; Awano et al., 2007), but deletion of *rhlE* did not affect growth at 37°C in *E. coli* (Phadtare, 2011).

Compatible Solutes

Our results show that *Y. pseudotuberculosis* expresses genes involved in glycine betaine intake and biosynthesis significantly more at 3°C during critical points of growth, i.e., the beginning and logarithmic phase. It has been shown that *Y. enterocolitica*, with almost identical *proU* and *proP* operons, accumulates glycine betaine at low temperature, but glycine betaine only protects it against osmotic stress (Park et al., 1995; Annamalai and Venkitanarayanan, 2009). However, glycine betaine has been shown to also grant cold protection to gram-positive *L. monocytogenes*, *B. subtilis*, and gram-negative *Vibrio anguillarum* (Ko et al., 1994; Hoffmann and Bremer, 2011; Ma et al., 2017). Furthermore, choline/carnitine/betaine transporter gene of *C. botulinum* was upregulated in cold shock (Dahlsten et al., 2014). The precise
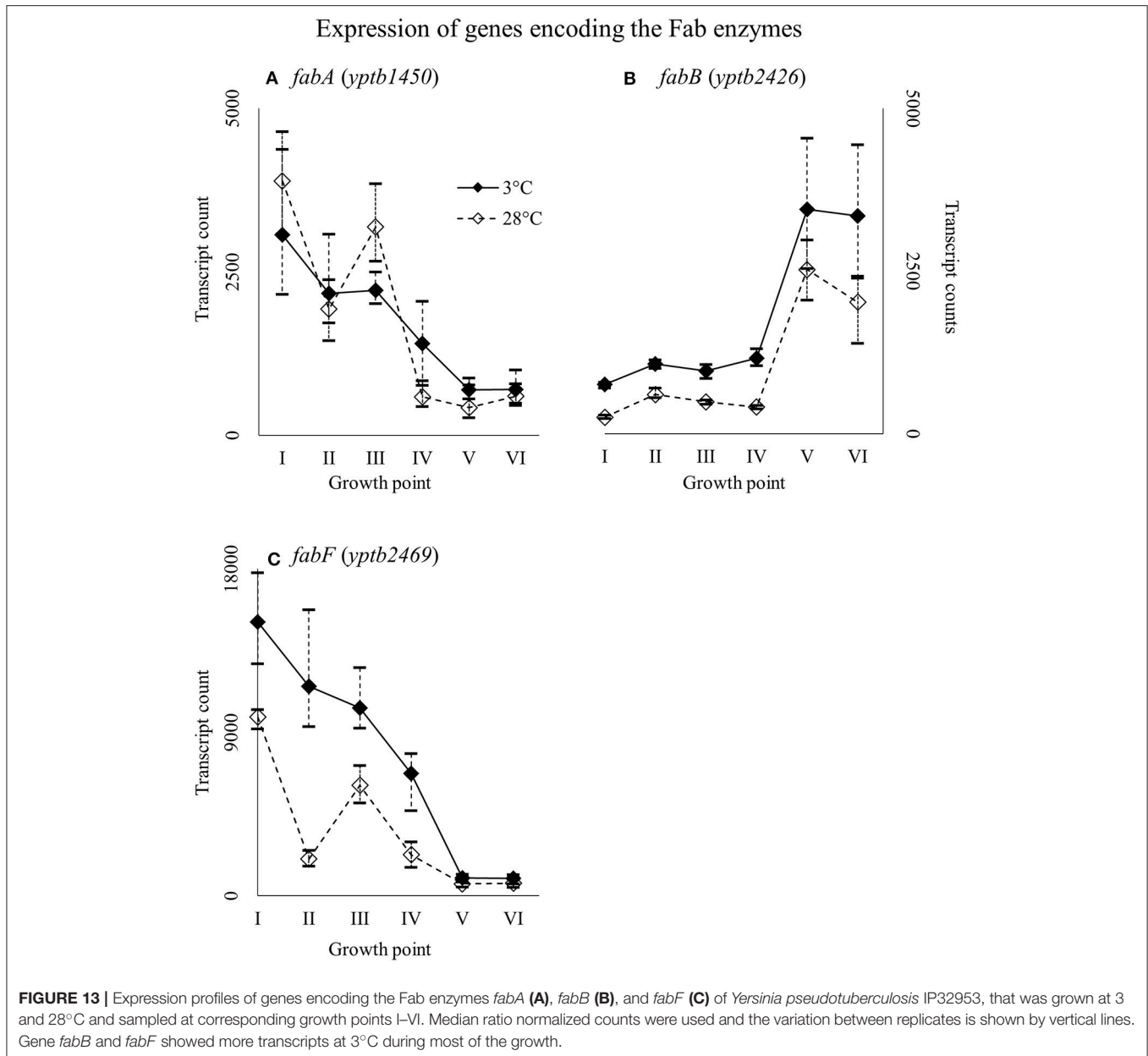
FIGURE 12 | Expression profiles of genes encoding transcription termination factor Rho **(A)**, translation initiation factor IF-1 **(B)**, and ribosome binding factor RbfA **(C)** of *Yersinia pseudotuberculosis* IP32953, that was grown at 3 and 28°C and sampled at corresponding growth points I–VI. Median ratio normalized counts were used and the variation between replicates is shown by vertical lines. All three factors showed more transcripts at 3°C at least to the end of logarithmic phase.

mechanism behind glycine betaine mediated cold protection is unclear, but the molecule has been suggested to hinder ice crystal formation and displace water around macromolecules, reducing aggregation and denaturation (Hoffmann and Bremer, 2011).

In our results, an operon encoding spermidine efflux pump MdtIJ was expressed significantly more at 3°C. Bacteria need spermidine for various purposes, but at low temperatures excess spermidine is detrimental (Higashi et al., 2008). Spermidine displaces magnesium-ions from ribosomes, thus deactivating them, but it also prolongs synthesis of Csps after cold shock (Limsuwun and Jones, 2000). It has been shown that prolonged synthesis of Csps after cold shock occupies all available ribosomes effectively stopping growth in *Y. enterocolitica* (Neuhaus et al.,

2000). Our results suggest that also *Y. pseudotuberculosis* ejects excess spermidine to avoid these problems.

Rho Factor, IF-1, and RbfA

Translation factors like IF-1 and RbfA act directly on ribosomes, securing protein synthesis. Initiation factor IF-1 separates the overly stabilized large and small subunits of ribosomes so that they can fuse and begin translating again elsewhere (Giangrossi et al., 2007), while RbfA primes 16S rRNA for new small subunits (Xia et al., 2003). Both IF-1 and RbfA have been shown to be significantly more expressed during cold shock in *E. coli* (Xia et al., 2003; Giangrossi et al., 2007). *E. coli* IF-1 resembles the CspA homologs (Phadtare et al., 2007), whereas in *Y.*

FIGURE 13 | Expression profiles of genes encoding the Fab enzymes *fabA* **(A)**, *fabB* **(B)**, and *fabF* **(C)** of *Yersinia pseudotuberculosis* IP32953, that was grown at 3 and 28°C and sampled at corresponding growth points I–VI. Median ratio normalized counts were used and the variation between replicates is shown by vertical lines. Gene *fabB* and *fabF* showed more transcripts at 3°C during most of the growth.

pseudotuberculosis they are only 42–50% similar in nucleic acid and 14–21% in amino acid sequence.

The *rho* gene is associated with bacteria that are subjected to various stresses in their environment (D'heygère et al., 2013). Rho factor is needed at low temperature to clear the bacterial DNA of frozen ribosomes and polymerases (D'heygère et al., 2013), and the structure of its RNA-binding subunit is similar to that of a cold shock domain (Briercheck et al., 1996). Rho factor also coordinates homeostasis of magnesium ions, which are needed by ribosomes, by controlling transporter genes (Kriner and Groisman, 2015). The Rho factor has been shown to be upregulated at low temperature in at least *Acidithiobacillus ferrooxidans* (Mykytczuk et al., 2011), *Pseudomonas haloplanktis* strain TAC125 (Piette et al., 2010), and *Bacillus subtilis* (Quirk

et al., 1993). To our knowledge, none of these factors have been linked to cold growth in *Yersinia* genus before.

Y. pseudotuberculosis expressed the genes encoding IF-1, RbfA, and Rho all through the critical phases of growth at high levels but also significantly more at 3°C. Massive difference of IF-1 (*yptb1395*) expression in favor of cold during logarithmic phase suggests that the initiation factor is important for continued growth at low temperature. It is probable that IF-1 and RbfA of *Y. pseudotuberculosis* provide the bacterium with working ribosomes at low temperature. *Y. pseudotuberculosis* might need Rho factor not only to terminate inefficient transcription but also to provide ribosomes with much needed magnesium ions. The upregulation of *mgt* transporter genes at 3°C throughout the growth supports this theory.

Cell Membranes

Lipid synthesis and cell growth cease in cyanobacteria until an adequate membrane lipid composition has been achieved (Sinetova and Los, 2016). However, gram-negative cyanobacteria and gram-positive *Bacillus subtilis* possess a two-component system, DesK-DesR, that senses increasing membrane rigidity at low temperatures (Beranová et al., 2010; Sinetova and Los, 2016). Although a corresponding two-component system has not been identified in *Y. pseudotuberculosis* (Palonen et al., 2011), the ratio of unsaturated to saturated fatty acids in its membranes rises at low temperature (Bakholdina et al., 2004). In another gram-negative bacterium, *E. coli*, temperature drop directly affects activity of the FabF enzyme in its cytosol and this enzyme is involved in unsaturated lipid synthesis with FabA and FabB (Mansilla et al., 2004). Cold stress has also been shown to induce the transcription of *fabF* in *Y. pestis* (Han et al., 2005).

It is also possible that the csp encoded by *yptb2414*, a homolog of *E. coli* CspC and CspE, is involved in cold adaptation of membranes in *Y. pseudotuberculosis*. CspC and CspE bind specific uracil-rich segments of mRNA, and these segments often encode hydrophobic structures of membrane proteins (Benhalevy et al., 2015). It has been suggested that bacteria use these segments as addresses for directing membrane protein mRNA to where they are needed in the cell (Benhalevy et al., 2015).

CONCLUSIONS

Y. pseudotuberculosis has extensive tools to keep its protein synthesis running at low temperature. It is probable, that the functions of its Csps, RNA helicases and factors acting on ribosomes overlap greatly and thus form a robust network to protect nucleic acids from cold damage. Csps encoded by *yptb1423*, *yptb2414*, *yptb2950*, *yptb3585–86,* and RNA helicases CsdA, RhlE, and DbpA, seem to form the backbone of cold survival of *Y. pseudotuberculosis* with their regulatory and nucleic acid unwinding functions. IF-1, RbfA, and by extension, Rho, keep the ribosomes of *Y. pseudotuberculosis* running even at suboptimal temperatures. Rho factor also terminates frozen ribosomes and RNA polymerases, freeing scant resources under cold stress.

Increased expression of motility, chemotaxis, and nutrient uptake genes at low temperature suggests that *Y. pseudotuberculosis* actively tries to find and secure sufficient resources to grow in refrigerator temperatures. The nutrients acquired are diverse and the nutrient profile seems to shift as growth progresses. The bacterium also possibly changes its membrane lipid composition with Fab enzymes, much like *E. coli,* to battle increasing membrane rigidity.

The exact role of glycine betaine in cold resistance is uncertain, but *Y. pseudotuberculosis* seems to accumulate it during cold growth like many other bacteria. Genes involved in defense against foreign DNA as well as oxidative stress were upregulated at 3°C, which would seem to suggest that stress responses are linked in some way.

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SUPPLEMENTARY MATERIAL

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