Global transcriptome analysis of Stenotrophomonas maltophilia in response to growth at human body temperature

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Abstract

Stenotrophomonas maltophilia is a typical example of an environmental originated opportunistic human pathogen, which can thrive at different habitats including the human body and can cause a wide range of infections. It must cope with heat stress during transition from the environment to the human body as the physiological temperature of the human body (37 °C) is higher than environmental niches (22–30 °C). Interestingly, S. rhizophila a phylogenetic neighbour of S. maltophilia within genus Stenotrophomonas is unable to grow at 37 °C. Thus, it is crucial to understand how S. maltophilia is adapted to human body temperature, which could suggest its evolution as an opportunistic human pathogen. In this study, we have performed comparative transcriptome analysis of S. maltophilia grown at 28 and 37 °C as temperature representative for environmental niches and the human body, respectively. RNA-Seq analysis revealed several interesting findings showing alterations in gene-expression levels at 28 and 37 °C, which can play an important role during infection. We have observed downregulation of genes involved in cellular motility, energy production and metabolism, replication and repair whereas upregulation of VirB/D4 type IV secretion system, aerotaxis, cation diffusion facilitator family transporter and LacI family transcriptional regulators at 37 °C. Microscopy and plate assays corroborated altered expression of genes involved in motility. The results obtained enhance our understanding of the strategies employed by S. maltophilia during adaptation towards the human body.

DATA SUMMARY

(1) Data file (.txt) for all replicates of two conditions i.e. three replicates at 28 °C and three replicates at 37 °C, used to generate the volcano plot obtained from the differential expression analysis (https://doi.org/10.6084/m9.figshare.14370461).

(2) Data file (.xlsx) used to generate the bar graph plot for the number of differentially expressed genes and number of CDS (https://doi.org/10.6084/m9.figshare.14370467).

(3) Data file (.xlsx) used for plotting the qRT-PCR bar graph for validation of the differentially expressed genes (https://doi.org/10.6084/m9.figshare.14375624).

(4) Data file (.xlsx) used for generating presence or absence heatmap of differentially expressed genes across the strains of Stenotrophomonas maltophilia complex (https://doi.org/10.6084/m9.figshare.14370470).

(5) Mapped reads for both conditions, i.e. at 28 and 37 °C ( _1, _2, and _3 represent the three replicates for each conditions) (https://doi.org/10.6084/m9.figshare.14370458).

INTRODUCTION

Variation in temperature is one of the most crucial stress factors for pathogens of environmental origin during adaptation to the human body, as temperature of the external biosphere is generally 22–30 °C. There are different molecular mechanisms by which bacteria sense and respond to changes in temperature. Moreover, temperature is one of the critical signals that influences the different bacterial processes. In bacterial pathogens of mammals including Shigella, Yersinia, Pseudomonas etc., the body temperature of the host, i.e. 37 °C induces the expression of virulence...
factors [1, 2]. Temperature is one of the important signals that a mammalian pathogen uses to regulate the virulence trait once it has entered its warm-blooded host [3]. In contrast, in pathogens of plants and ectothermic hosts such as fish, molluscs and amphibians, virulence gene expression is elevated at the lower temperatures, suggesting a role of temperature in the coordination of bacterial pathogenesis and virulence [4, 5]. Recently discovered RNA thermometers are an interesting tool in bacteria for responding to such external temperature stresses. They are RNA structures formed at the 5′ UTR regions of transcripts specifying regulatory proteins responsible for expression of virulence-associated traits, which blocks translation initiation of genes at non-permissive temperatures [6].

Genus *Stenotrophomonas* comprises several species from a diverse range of niches such as *S. lactitubi* and *S. indicatrix* from food [7], *S. bentonitica* [8] and *S. chelatiphaga* from soil etc. [9–11]. *S. maltophilia* is a ubiquitous bacterium, which has emerged as a multidrug-resistant global opportunistic pathogen in immunocompromised patients [12–14]. *S. maltophilia* is a versatile bacterium, which adapts to a wide range of environments and it is the only validated species among *Stenotrophomonas* genus, which causes human- and animal-associated infections [9, 15]. Apart from this detrimental effect, *S. maltophilia* has an extraordinary range of activities such as plant growth promotion, degradation of anthropogenic pollutants and production of biomolecules [15, 16]. Presence of such a wide range of properties makes this bacterium an important biotechnological candidate, but the pathogenic potential of this bacterium limits its use for biotechnological applications [16]. The comparison of the *S. maltophilia* with *S. rhizophila*, a non-pathogenic and phylogenetically related species, revealed that *S. rhizophila* lacks crucial virulence factors and heat-shock proteins [17]. *S. rhizophila* is unable to grow at human body temperature, 37 °C due to the absence of heat-shock genes and upregulation of genes involved in suicidal mechanisms [17]. Thus, it is essential to understand the adaptation of rapidly emerging multidrug-resistance opportunistic pathogen *S. maltophilia* to human body temperature, which is considered as the first step towards transition from environment to the human body.

Advances in high-throughput sequencing approaches will accurately quantify levels of expression of mRNA (RNA-Seq) thus, providing significant advances over microarrays [18–20]. To understand the genetic response, mechanistic basis and factors involved in the successful adaptation of the *S. maltophilia* at human body temperature, we systematically examined the transcriptome during the growth at 28 and 37 °C using RNA-Seq experiments.

**Impact Statement**

*Stenotrophomonas maltophilia* is a WHO listed multidrug-resistant nosocomial pathogen. Interestingly, *S. maltophilia* species can grow both at 28 and 37 °C unlike its closest taxonomic relative, i.e. *S. rhizophila* and also the majority species belonging to this genus. Hence this ability to grow at 37 °C, i.e. human body temperature might have played a key role in the unique success and emergence of this species as an opportunistic human pathogen. Using transcriptome sequencing, we have identified a set of genes, which are differentially regulated at 37 °C, and investigated their evolutionary history. This study has revealed regulation of genes involved in motility, metabolism, energy, replication, transcription, aerotaxis and a type IV secretion system might have a role in successful adaption to a distinct lifestyle. The findings will be helpful in further systematic studies on understanding and management of an emerging human pathogen such as *S. maltophilia*.

ATCC 13637T was grown in Luria–Bertani Miller Broth with shaking at 200 r.p.m. at either 37 or 28 °C.

**Total RNA extraction, quantification and Integrity estimation**

*S. maltophilia* ATCC 13637T was grown in 20 ml Luria–Bertani Broth, Miller in 100 ml Erlenmeyer flask at 37 and 28 °C under constant agitation at 200 r.p.m. Samples were withdrawn at intervals for optical density monitoring at 600 nm (OD600), and cells from both cultures were harvested at mid-log phase (OD600 =0.8 to 1) by centrifugation at 6000 r.p.m. at for 10 min at 4 °C and immediately frozen at −80 °C or proceeded to the RNA isolation. For isolation of RNA, the pellet was suspended in the 1 ml of TRizol (Invitrogen, Carlsbad, CA, USA) and dissolved by vigorous mixing. The supernatant was transferred into a clean tube, which contained one volume of 100% ethanol mixed by repeated gentle inversion. The RNA was purified and treated with DNase by using the Direct-zol RNA MiniPrep kit (Zymo Research Corporation, Orange, CA, USA), according to the manufacturer’s recommendation. The purity of isolated total RNA, was determined by using the NanoDrop (Thermo Scientific, Wilmington, DE, USA) and quantified by using Qubit (Invitrogen, Carlsbad, CA, USA). Agilent Bioanalyzer with Agilent RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA, USA) was used as per manufacturer’s guidelines to assess the integrity of RNA samples. The RNA samples with RNA Integrity Number (RIN) >8 were selected for cDNA synthesis and subsequent Illumina library construction.

**Ribosomal RNA depletion, cDNA library preparation and Illumina sequencing**

The ScriptSeq complete kit (Epicentre, Illumina, Madison, WI, USA), a combined kit for the ribosomal (rRNA) depletion
Table 1. List of primers used in qRT-PCR for validation of RNA-Seq
genes by using ScriptSeq Index PCR Primers (EpigenTech, Illumina, Madison, WI, USA) and cDNA library preparation kit (ScriptSeq v2 RNA-Seq library preparation kit (EpigenTech, Illumina, Madison, WI, USA)) was used for this purpose. A total of 5µg of RNA was used for rRNA depletion by using Ribo-Zero (Epicentre, Madison, WI, USA) and further used for the cDNA synthesis using ScriptSeq Index PCR Primers (EpigenTech, Illumina, Madison, WI, USA) and multiplexed Ribo-Zero Kit (Bacteria) (EpigenTech, Illumina, Madison, WI, USA) and cDNA library preparation kit, ScriptSeq v2 RNA-Seq library preparation kit (EpigenTech, Illumina, Madison, WI, USA) was used for this purpose. A total of 5µg of RNA was used for rRNA depletion by using Ribo-Zero (Epicentre, Madison, WI, USA) and cDNA library construction kit, ScriptSeq v2 RNA-Seq kit (Epicentre, Illumina, Madison, WI, USA) was used for this purpose. A total of 5 µg of RNA was grown at 28 °C as described earlier. The quantitation of the differentially expressed genes was designed by using primer3 tool (http://bioinfo.ut.ee/primer3-0.4.0/) and listed in Table 1. RNA was isolated from bacterial cells cultured at 28 °C (SM_28_R1, SM_28_R2, SM_28_R3) and 37 °C (SM_37_R1, SM_37_R2, SM_37_R3) were pooled and sequenced using in-house Illumina MiSeq (Illumina, San Diego, CA, USA) platform with 2×75 bp paired end run.

RNA-Seq data analysis
The indexing adapters were trimmed by MiSeq control software during the base calling and read quality assessment was done using FastQC v0.11.2 [21]. The complete genome sequences of *S. maltophilia* ATCC 13637 (accession no.: NZ_CP008838) was downloaded from NCBI-GenBank (https://www.ncbi.nlm.nih.gov/genome/880?genome_assembly_id=205295) and used as a reference for aligning the reads by using Bowtie2 [22]. The aligned SAM files generated by bowtie were sorted using samtools v1.4.1 [23]. The obtained BAM files were used as input to *cufflinks* v2.2.1 [19, 24, 25], which was used to assemble transcripts with FPKM (fragments per kilobase of transcript per million mapped reads) values. The data files for the replicates were merged into single transcript with *Cuffmerge* and differential gene expression analysis between both conditions, i.e. 28 and 37 °C was performed using the *Cuffdiff*, a package of the *cufflinks* v2.2.1 [19, 24, 25]. The output data from *Cuffdiff* were imported to *cummeRbund* v2.32 [26], which is based on R statistical package version 3.4.0 for visualization. Gene-expression data were deposited to the Gene Expression Omnibus database (accession number: GSE101926).

qRT-PCR validation of the differentially expressed genes
To confirm some of the differentially expressed genes obtained using RNA-Seq, a conventional real-time quantitative reverse transcription-PCR (qRT-PCR) was employed to measure changes in the mRNA level of each gene. Gene-specific primers of the differentially expressed genes were designed by using primer3 tool (http://bioinfo.ut.ee/primer3-0.4.0/) and listed in Table 1. RNA was isolated from bacterial cells grown at 28 and 37 °C as described earlier. The quantitative real-time PCR assay was performed with SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Thermo Scientific, Wilmington, DE, USA). For each sample, three technical replicates were included, and reactions were set up according to the manufacturer's guidelines. The amplification conditions were as follows: cDNA synthesis 50 °C for 45 min, initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 15 s followed by annealing at 60 °C for 30 s and extension at 40 °C for 30 s. Melting curve analysis confirmed that all PCRs amplified a single product. Gene-expression levels were normalized to 16S rRNA gene and *fsZ* gene. The relative expression of each gene at 37 °C relative to 28 °C was expressed as fold change calculated by using 2^ΔΔct method. The resulting fold-change values were

| S. no. | Gene | Primer sequence (5′−3′) |
|-------|------|------------------------|
| 1     | SM-fsz-Z-F | GGCAGGTTTGGAACTGGTCG |
|      | SM-fsz-Z-R | AGCTGGACCCGGCAATCTT |
| 2     | SM-fimA-F | TGCCGACGGTGGTCAAGAA |
|      | SM-fimA-R | GCACCTGGTCAGGTGAGGG |
| 3     | SM-fimB-F | ACTTGGCGGAGTATGACG |
|      | SM-fimB-R | GCCGTAGTCGCTGGATGGTGA |
| 4     | SM-16s-F | GACCTTGCGCGATTGAGT |
|      | SM-16s-R | CGGATCCTGGCCTGGTAT |
| 5     | SM-virB1-F | GTGCAGGTCGGACACATCC |
|      | SM-virB1-R | GATGGTAAACGGTGAGG |
| 6     | SM-virB4-F | TGTTGATGGACAAATCTGGA |
|      | SM-virB4-R | ATCACTCTTCACGCCGCTT |
| 7     | SM-virB6-F | GTGGCGATGTCGATGGTAT |
|      | SM-virB6-R | AATCGCGTAAAGACAGCCAC |
| 8     | SM-virB11-F | CGGCGATACGCAAGTCTTT |
|      | SM-virB11-R | TCTGGTCGGGCGAGATGAT |
| 9     | SM-trbJ-F | CATGACATCCGAAATCATCA |
|      | SM-trbJ-R | GTGGCGAGGAGAGGGTAACCA |
| 10    | SM-MMT12-F | CAGCAATCCATGGTCTCGA |
|      | SM-MMT12-R | CATCGACGTCGACACAGAGATGTT |
| 11    | SM-yehB-F | CAGTTCAACTCCAGCTTCTTG |
|      | SM-yehB-R | AGCTACACGTGCAACAGATGAT |
| 12    | SM-fruF-F | GATTGTCGAGTACACGGCTG |
|      | SM-fruF-R | CACGTATCTGCAATTGATGAC |
| 13    | SM-aer-F | GTATACAGGAGATTGGAAGC |
|      | SM-aer-R | GATGCTGATGAAAGGTAGT |
| 14    | SM-cspA2-F | GGAACGCTTGTTCGACCTCTC |
|      | SM-cspA2-R | GTCAGCCTGCAATACCCCTTCT |
Functional categorization of differentially expressed genes

EggNOG v4.5.1, a database [27] of orthologous groups and functional annotation was used to classify genes differentially expressed at 28 and 37 °C into functional categories based on Clusters of Orthologous Groups (COG).

All the full-length differentially expressed genes obtained from the RNA-Seq experiment were fetched from all the type strains of genus *Stenotrophomonas maltophilia* complex (Smc) using *tblastn* [28]. Cut-off for similarity was set to be 60% and coverage was 50%. All the differentially expressed genes from reference genome ATCC 13637T were annotated using eggNOG-mapper v2 [27]. Based on the presence and absence of the gene a heatmap was constructed using GENE-Ev3.0.215 (https://software.broadinstitute.org/GENE-E/).

Transmission electron microscopy

Transmission electron microscopy was used to visualize the morphology of the flagella at 28 and 37 °C. Bacterial cultures were grown in 20 ml LB and incubated at 28 and 37 °C, respectively, until OD600nm reaches 0.8. The cells were harvested by centrifugation at 2000 r.p.m. for 10 min. The cell pellet was washed twice with 1X PBS (Invitrogen, Carlsbad, CA, USA) and finally suspended in 50μl of 1X PBS (Invitrogen, Carlsbad, CA, USA). Altogether, 10–20 μl of bacterial suspension was placed on a carbon-coated copper grid (300 mesh, Nissin EM) for 15 min. The grid was then negatively stained for 30 s with 2% phosphotungstic acid, dried and examined using JEM 2100 transmission electron microscope (JEOL, Tokyo, Japan) operating at 200 kV.

Motility assays

Motility patterns of *Stenotrophomonas maltophilia* ATCC 13637T were assessed by using motility media. For swimming motility, 5 μl of overnight grown culture was spotted on plates containing 1% tryptone, 0.5% NaCl and 1.2% agar. A bacterial colony was spotted on plates containing 1% tryptone, 0.5% NaCl and 0.3% agar. Similarly, for swarming motility 5 μl of overnight grown culture was spotted on plates containing 1% tryptone, 0.5% NaCl and 0.5% agar. Plates were incubated at 28 and 37 °C for 7 days. Then, to check twitching motility, agar was removed, and plates were stained with 0.1% crystal violet. Motility assays were carried out on three biological replicates.

Growth curve measurements

The growth curves at two temperatures, i.e. 28 and 37 °C was generated by growing bacterial culture at 28 and 37 °C overnight. Then, 1% of the overnight grown culture (OD=1.0) was then inoculated in fresh 50 ml LB with an initial OD<sub>600</sub> nm 0.015. Readings were taken every 1 h for 32 h at OD<sub>600</sub> nm.

RESULTS AND DISCUSSION

Comparative transcriptome analyses of *S. maltophilia* during growth at 28 and 37 °C

To determine the genetic mechanism underlying adaptation of *S. maltophilia* at human body temperature, we performed RNA-Seq analysis on three biological replicates of *S. maltophilia* grown at 28 and 37 °C.

A total 4676670, 9477113, 7989000 and 3536078, 11310235 and 14241935 paired-end sequencing reads were obtained for three biological replicates for growth at 28 °C (SM_28_R1, SM_28_R2, SM_28_R3) and 37 °C (SM_37_R1, SM_37_R2, SM_37_R3), respectively. Reads from all replicates were mapped to the reference genome *S. maltophilia* ATCC 13637T with overall mapping frequency ranging from 87–94% (Table 2).

To identify differentially expressed genes at 37 °C, we compared transcript profiles of *S. maltophilia* ATCC 13637T grown at 28 and 37 °C. The global transcriptional profiles for two conditions were obtained by data normalization and statistical analysis. A matrix of pairwise comparison based on the FPKM values between two conditions was obtained. It was used to generate the volcano plot (Fig. 1a) to map the fold change in transcript expression against its statistical significance (q-values).

Total 81 gene were differentially expressed when the *S. maltophilia* ATCC 13637T was grown at 37 °C as compared to growth at 28 °C with the statistically significant cut-off values: q-value <0.05 and log<sub>2</sub>-fold change >2. Also, all the hypothetical and ribosomal genes were removed from the list of differentially expressed genes. Among differentially expressed genes, 12 genes (accounting for the 15% of differentially expressed genes) were upregulated (Table 3) while 69 genes (accounting for 85%) were downregulated at 37 °C as compared to the 28 °C (Table 4). The classification of differentially expressed genes by COG revealed that genes in 16 COG classes were differentially expressed (Fig. 1b). The differentially expressed genes were differentially expressed are intracellular trafficking and secretion, signal transduction, cell motility and with unknown function (Fig. 1b). The differentially
expressed genes belonging to the cell motility; secondary structure; post-translational modification; replication and repair; translation; lipid metabolism; coenzyme metabolism; nucleotide metabolism and transport; amino acid metabolism and transport classes were downregulated at 37 °C.

To partially validate the differentially expressed genes during growth at 37 °C as compared to at 28 °C, we performed the qRT-PCR analysis of selected genes. We have analysed the expression profiles of 12 randomly selected differentially expressed genes at 37 and 28 °C (Fig. 2). We have used 16S rRNA and ftsZ genes as an internal control.

Table 3. S. maltophilia ATCC 13637T genes significantly up-regulated during the growth at 37 °C versus 28 °C

| Locus tag     | Gene description                             | Gene name | log₂-fold change | P-value | q-value |
|---------------|----------------------------------------------|-----------|------------------|---------|---------|
| DP16_RS07185  | VirB4 family type IV secretion/conjugal transfer ATPase | virB4     | 2.96759          | 0.00005 | 0.00022 |
| DP16_RS09460  | PAS sensor domain-containing protein and sulfite reductase subunit alpha | aer       | 3.9106           | 0.00005 | 0.00022 |
| DP16_RS07200  | Type IV secretion protein                    | virB1     | 2.72586          | 0.00005 | 0.00022 |
| DP16_RS07180  | trbJ                                         | trbJ      | 2.64674          | 0.00005 | 0.00022 |
| DP16_RS07205  | P-type DNA transfer ATPase VirB11            | virB11    | 2.28306          | 0.00005 | 0.00022 |
| DP16_RS10420  | Transcriptional regulator, LacI family       | fruR      | 2.23076          | 0.00005 | 0.00022 |
| DP16_RS07175  | Type IV secretion protein                    | virB6     | 2.2604           | 0.00005 | 0.00022 |
| DP16_RS06915  | Cation transporter                           | MMT12     | 2.01605          | 0.00005 | 0.00022 |
| DP16_RS14080  | MULTISPECIES: type IV plus modification protein PilV | PilV      | 2.06625          | 0.0007  | 0.00241 |
| DP16_RS14085  | MULTISPECIES: Tlp plus assembly protein FimT/FimU | Tlp       | 2.14686          | 0.00135 | 0.00429 |
| DP16_RS07215  | MULTISPECIES: TrbG/VirB9 family P-type conjugative transfer protein | TrbG      | 2.36236          | 0.00005 | 0.00022 |
| DP16_RS07195  | MULTISPECIES: TrbC/VirB2 family protein      | TrbC      | 2.76037          | 0.00005 | 0.00022 |
Table 4. *S. maltophilia* ATCC 13637\(^\text{T}\) genes significantly down-regulated during growth at 37 °C versus 28 °C

| Locus tag          | Gene description                                                                 | Gene name       | log\(_2\)-fold change | P value   | q-value   |
|-------------------|----------------------------------------------------------------------------------|-----------------|-----------------------|-----------|-----------|
| DP16_RS19065      | Fimbrial biogenesis outer membrane usher protein/Type one fimbrial protein       | mrkD, yehB      | −3.96419              | 0.00005   | 0.00022   |
| DP16_RS19075      | Fimbrial protein                                                                  | fimA            | −4.32102              | 0.00005   | 0.00022   |
| DP16_RS19070      | Fimbrial chaperone                                                                | fimB            | −3.74341              | 0.00005   | 0.00022   |
| DP16_RS01055      | Porin                                                                            | −               | −3.26817              | 0.00005   | 0.00022   |
| DP16_RS15085      | Type I methionyl aminopeptidase                                                   | map             | −2.97022              | 0.00005   | 0.00022   |
| DP16_RS03855      | Chemotaxis protein                                                                | mcpU            | −2.60711              | 0.00005   | 0.00022   |
| DP16_RS16150      | Polypeptide cyclase                                                               | −               | −2.46992              | 0.00005   | 0.00022   |
| DP16_RS08430      | beta-hydroxydecanoyl-ACP dehydratase/beta-ketoacyl-[acyl-carrier-protein] synthase I | fabA, fabB     | −2.81952              | 0.00005   | 0.00022   |
| DP16_RS11160      | Flagellin                                                                        | fliC            | −2.34589              | 0.00005   | 0.00022   |
| DP16_RS12455      | Short-chain alcohol dehydrogenase family                                         | −               | −2.57592              | 0.00005   | 0.00022   |
| DP16_RS01020      | C4-dicarboxylate transporter                                                      | dctA            | −2.31709              | 0.00005   | 0.00022   |
| DP16_RS21325      | Methyl-accepting chemotaxis protein                                              | mcpU            | −2.31383              | 0.00005   | 0.00022   |
| DP16_RS01805      | ATP synthase subunit B                                                            | atpF            | −2.62816              | 0.00005   | 0.00022   |
| DP16_RS12245      | Ribosome biogenesis GTPase Der                                                    | der             | −2.23516              | 0.00005   | 0.00022   |
| DP16_RS02060      | Peptidase M28 family protein                                                      | −               | −2.16125              | 0.00005   | 0.00022   |
| DP16_RS20730      | Peptidyl-prolyl cis-trans isomerase                                               | sylDB           | −2.49939              | 0.00005   | 0.00022   |
| DP16_RS21125      | DsbA family oxidoreductase                                                        | fnrE            | −2.21017              | 0.00005   | 0.00022   |
| DP16_RS17970      | Translational GTPase TypA                                                         | typA            | −2.07728              | 0.00005   | 0.00022   |
| DP16_RS02290      | Iron-uptake factor                                                                | −               | −2.11432              | 0.00005   | 0.00022   |
| DP16_RS06505      | Transamidase GaB domain protein                                                   | yqeY            | −2.84454              | 0.00005   | 0.00022   |
| DP16_RS11100      | Chemotaxis protein CheV                                                           | cheV2           | −2.14625              | 0.00005   | 0.00022   |
| DP16_RS12425      | Cold-shock protein                                                                | cspA2           | −4.00154              | 0.00005   | 0.00022   |
| DP16_RS02690      | S-adenosylmethionine decarboxylase proenzyme                                       | speD            | −2.42359              | 0.00005   | 0.00022   |
| DP16_RS04645      | Biopolymer transporter ExbB                                                       | exbB            | −2.002                | 0.00005   | 0.00022   |
| DP16_RS02840      | Dihydroorotate dehydrogenase                                                      | dtpA            | −2.11169              | 0.00005   | 0.00022   |
| DP16_RS09945      | Mg(2+) transport ATPase C                                                         | mgtC            | −2.01719              | 0.00005   | 0.00022   |
| DP16_RS16825      | BolA family transcriptional regulator                                              | −               | −4.09326              | 0.00005   | 0.00022   |
| DP16_RS06005      | Entericidin A/B family lipoprotein                                                | −               | −4.0634               | 0.00005   | 0.00022   |
| DP16_RS20940      | RidA family protein                                                               | −               | −3.23711              | 0.00005   | 0.00022   |
| DP16_RS12470      | Preprotein translocase subunit YajC                                               | yajC            | −2.69781              | 0.00005   | 0.00022   |
| DP16_RS19265      | Ferredoxin–NADP reductase                                                         | −               | −2.6871               | 0.00005   | 0.00022   |
| DP16_RS21105      | Cold-shock protein                                                                | −               | −2.54197              | 0.00005   | 0.00022   |
| DP16_RS11430      | AbrB/MaeE/SpoVT family DNA-binding domain-containing protein                       | −               | −2.53552              | 0.0001    | 0.00042   |
| DP16_RS05720      | Sulphate transporter                                                              | −               | −2.53105              | 0.0011    | 0.0036    |
| DP16_RS21940      | YebC/PmpR family DNA-binding transcriptional regulator                            | −               | −2.50375              | 0.00005   | 0.00022   |

Continued
| Locus tag   | Gene description                      | Gene name | log₂-fold change | P value  | q-value |
|------------|---------------------------------------|-----------|------------------|----------|---------|
| DP16_RS13530 | Carbon storage regulator CsrA         | csrA      | −2.46402         | 0.00005  | 0.00022 |
| DP16_RS00255 | Large-conductance mechanosensitive channel protein MscL | mscL      | −2.45568         | 0.00005  | 0.00022 |
| DP16_RS11010 | Translation initiation factor IF-1    | infA      | −2.43986         | 0.00005  | 0.00022 |
| DP16_RS07410 | NAD(P)H-dependent oxidoreductase       |           | −2.39581         | 0.00005  | 0.00022 |
| DP16_RS19725 | Polyhydroxyalkanoate synthesis repressor PhaR | phaR      | −2.3836          | 0.00005  | 0.00022 |
| DP16_RS06535 | DNA-directed RNA polymerase subunit omega | rpoZ      | −2.37859         | 0.00005  | 0.00022 |
| DP16_RS16300 | Nucleoside hydrolase                  |           | −2.37001         | 0.00005  | 0.00022 |
| DP16_RS17255 | Acyl carrier protein                  | acpP      | −2.36121         | 0.00005  | 0.00022 |
| DP16_RS23905 | Helix-turn-helix transcriptional regulator |         | −2.35943         | 0.00005  | 0.00022 |
| DP16_RS11090 | Flagellar biosynthesis anti-sigma factor FlgM | flgM      | −2.25741         | 0.00005  | 0.00022 |
| DP16_RS18345 | Asparaginase                          |           | −2.25033         | 0.00005  | 0.00022 |
| DP16_RS02380 | Type II 3-dehydroquinate dehydratase   | aroQ      | −2.24895         | 0.00005  | 0.00022 |
| DP16_RS11380 | Chemotaxis protein CheW               |           | −2.23436         | 0.00005  | 0.00022 |
| DP16_RS08700 | Metal-sensing transcriptional repressor |           | −2.23201         | 0.0015   | 0.00469 |
| DP16_RS17330 | YbaB/EbfC family nucleoid-associated protein |         | −2.16749         | 0.00005  | 0.00022 |
| DP16_RS14345 | PepSY domain-containing protein        |           | −2.15168         | 0.00005  | 0.00022 |
| DP16_RS13550 | Transcriptional repressor LexA         | lexA      | −2.13903         | 0.00005  | 0.00022 |
| DP16_RS20565 | VOC family protein                    |           | −2.13776         | 0.00005  | 0.00022 |
| DP16_RS20980 | YkgJ family cysteine cluster protein   |           | −2.1328          | 0.00005  | 0.00022 |
| DP16_RS04035 | S9 family peptidase                   |           | −2.11185         | 0.00005  | 0.00022 |
| DP16_RS06305 | Potassium-transporting ATPase subunit F |           | −2.10327         | 0.00005  | 0.00022 |
| DP16_RS22820 | Ribonuclease P protein component       |           | −2.09672         | 0.0024   | 0.00703 |
| DP16_RS00680 | Tetratricopeptide repeat protein       |           | −2.0782          | 0.00005  | 0.00022 |
| DP16_RS18560 | 16S rRNA (adenine(1518)-N(6)/adenine(1519)-N(6))-dimethyltransferase RsmA | rsmA      | −2.07383         | 0.00005  | 0.00022 |
| DP16_RS12255 | Tetratricopeptide repeat protein       |           | −2.05998         | 0.00005  | 0.00022 |
| DP16_RS05260 | Protein-export chaperone SecB          | secB      | −2.04198         | 0.00005  | 0.00022 |
| DP16_RS23910 | Helix-turn-helix transcriptional regulator |         | −2.03053         | 0.00005  | 0.00022 |
| DP16_RS03195 | Antibiotic biosynthesis monoxygenase   |           | −2.02961         | 0.00005  | 0.00022 |
| DP16_RS14630 | Elongation factor Ts                   |           | −2.02609         | 0.00005  | 0.00022 |
| DP16_RS21410 | Type II toxin-antitoxin system ParD family antitoxin |         | −2.02584         | 0.00005  | 0.00022 |
| DP16_RS13440 | H-NS histone family protein            |           | −2.01906         | 0.00005  | 0.00022 |
| DP16_RS21690 | 3′\(\(2\(\(5\)\))\)bisphosphate nucleotidase CysQ | cysQ      | −2.01471         | 0.00005  | 0.00022 |
| DP16_RS12275 | Nucleoside-diphosphate kinase          | ndk       | −2.01154         | 0.00005  | 0.00022 |
| DP16_RS12330 | (d)CMP kinase                         |           | −2.011           | 0.00005  | 0.00022 |
High correlation (R²=0.9135) between expression levels of genes measured by the RNA-Seq and qRT-PCR was observed (Fig. 2).

**Temperature-dependent regulation of cell motility**

The majority of the differentially expressed genes belong to the cell-motility category, and all of them were downregulated at 37 °C. These include isoforms of a gene (DP16_RS19060, DP16_RS19065), which encodes for fimbrial outer-membrane protein and type I fimbrial proteins, fimbrial proteins (DP16_RS19075), fimbrial chaperone (DP16_RS19070), methyl-accepting chemotaxis (DP16_RS03855), flagellin (DP16_RS11160), methyl-accepting chemotaxis protein (DP16_RS21325) and CheV chemotaxis protein (DP16_RS11100). The methyl-accepting chemotaxis proteins and CheV chemotaxis protein are categorized into signal transduction class along with GTP-binding protein TypA (DP16_RS17970), which were also downregulated (Table 4).

In order to check the phenotypic effect of downregulation of the cell motility and chemotaxis genes at 37 °C, we have performed the swimming and swarming motility assay during growth at 28 and 37 °C. The swimming and swarming motility is affected at 37 °C as compared to that of 28 °C (Fig. 3a, b). Further, downregulation of genes involved in flagellin biosynthesis leads to the development of less or impaired flagella at 37 °C as compared to the 28 °C, which was observed in transmission electron microscopy (Fig. 3c). The impaired flagella ultimately affect the motility at 37 °C as compared to the 28 °C. Taken together, these observations suggest the thermoregulation of cell motility in *S. maltophilia*.

In bacterial pathogens, it is now a well-known fact that virulence-related traits are generally overexpressed at physiological temperature, i.e. 37 °C [3]. The repression of motility genes at 37 °C to avoid the host recognition was also reported in *Listeria monocytogenes*, which is a food-borne pathogen of environmental origin [29]. In *Listeria monocytogenes*, mogR transcriptional repressor of flagellar genes along with a protein thermometer gmaR, which represses flagellar biosynthesis at 37 °C. The temperature-dependent regulation of motility was observed in several human and plant pathogens like *Yersinia enterocolitica*, *Listeria monocytogenes*, *Escherichia coli* and *Pseudomonas syringae* [30–33].

The flagella and fimbriae serve as pattern recognition molecule (PAMP), which activate innate immune response in the host cell, thus acting as an essential virulence factor for *S. maltophilia* [34]. Despite the important role of flagellin and fimbria genes in the *S. maltophilia* pathogenesis, these genes were downregulated at 37 °C suggesting that it is an adaptive mechanism by which *S. maltophilia* avoids host recognition and subsequent host innate immune response. In *S. maltophilia FsnR* is a canonical positive regulator directly or indirectly controlling the transcription of most flagellar genes by binding to the promoter region of the flagellar biosynthesis gene cluster [35]. There might be an involvement of the unidentified protein thermometer,
Fig. 3. Temperature-dependent regulation of motility. (a) Twitching motility of *S. maltophilia* ATCC 13637T observed during growth at 28 and 37 °C. (b) Swarming motility of *S. maltophilia* ATCC 13637T observed at growth 28 and 37 °C. (c) Transmission electron micrographs of *S. maltophilia* ATCC 13637T grown at 28 and 37 °C on nutrient agar and negatively stained with 1% phosphotungstic acid.

Fig. 4. Growth-curve measurement. Growth curve of *S. maltophilia* ATCC 13637T at two temperatures, i.e. 28 and 37 °C.
Fig. 5. Heatmap showing the presence or absence of differentially expressed genes in Smc along with log₂ fold change of the genes at 37 °C as compared to 28 °C. Genes related to (a) metabolism, (b) information storage and processing, (c) cellular processing and signalling, (d) others.
which along with the FsnR may regulate the temperature-dependent flagellar motility. The chemotaxis involves selective movements by using flagella and pili towards nutrients or to escape from hostile environments. There is downregulation of the multiple key genes involved in chemotaxis, which is in accordance with the downregulation of the flagellin genes.

**Downregulation of genes involved in energy production, metabolism and protein synthesis**

The expression of two genes involved in energy production and conservation were downregulated at 37 °C. These include the ATP synthase subunit beta (DP16_RS01805) and C4-dicarboxylate transporter (DP16_RS01020) responsible for uptake of fumarate, succinate and malate, which are essential for Pseudomonas aeruginosa and relatively less successful opportunistic human pathogens like Burkholderia cepacia. Downregulated genes belong to inorganic ion transport and metabolism category, including phosphate-selective porin O and P (DP16_RS01055), iron uptake factor (DP16_RS02290). The data suggested that downregulation of two genes involved in translation (DP16_RS15085), which encodes for a protein that removes the N-terminal methionine from nascent proteins. The genes belonging to COG class: post-translational modification, protein turnover, chaperone functions DP16_RS02730 (peptidylprolyl cis-trans isomerase), nucleotide metabolism and transport, DP16_RS12245 (ribosome biogenesis GTPase), amino acid metabolism and transport, DP16_RS02690 (S-adenosylmethionine decarboxylase proenzyme), DP16_RS02840 (Dihydroporotate dehydrogenase), energy production and conversion DP16_RS01020 (sodium dicarboxylate symporter family), DP16_RS01805 (ATP synthase subunit B) were also downregulated. The downregulation of genes involved in energy production and metabolism; translation is reflected in the lower growth rate of Stenotrophomonas maltophilia ATCC 13637T at 37 °C as compared to 28 °C (Fig. 4). This also suggests a reduction in energy production processes in Stenotrophomonas maltophilia ATCC 13637T may represent a survival strategy during adaptation at human body temperature.

As *S. maltophilia* is an emerging nosocomial pathogen and not a ‘professional pathogen’, the effect of flagella may not be for infection purpose per se. For example, when it attaches equipment used in hospital settings and on patients and in biofilm form, downregulation of flagella and hence motility is expected and required. Similarly, other selective forces besides body temperature may be playing a role in altering the expression of genes in *S. maltophilia*. This can be seen in dry/high temperatures seen during summers and its lifestyle as soil/water in-habitant where temperature fluctuations are through the year. Hence such non-human-body-induced temperature changes may be counterproductive as seen in downregulated energy metabolism and may be one of the reasons for its status as an emerging and relatively less successful opportunistic human pathogens like *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex.

**Upregulation of VirB/D4 type IV secretion system at 37 °C**

Comprehensive functional and COG analyses of upregulated genes revealed that five pivotal genes DP16_RS07185/virB4, DP16_RS07180/trbF, DP16_RS07200/virB1, DP16_RS07205/virB11 and DP16_RS07175/virB6 that are part of the type IV VirB/D4 secretory system, were upregulated at 37 °C (Table 3). The expression of the VirB/D4 T4SS components *virB4, trbF, virB1, virB11* and *virB6* was higher at 37 °C suggesting that VirB/D4 T4SS in *S. maltophilia* ATCC 13637T is regulated by the temperature. T4SS in *S. maltophilia* is horizontally acquired and present on the genomic island. It is present in the eight other non-clinical species of genus *Stenotrophomonas*, i.e. *S. chelatiphaga*, *S. daejeonensis*, *S. ginsengisoli*, *S. indicatrix*, *S. koreensis*, *S. lactitubi*, *S. pavanii* and *S. pictorum* [36]. The VirB/D4 T4SS is absent in the *S. acidaminiphila*, *S. nitritireducens*, *S. panacihumi*, *S. rhizophila* and *S. terrae* [36]. Apart from the role in conjugation, T4SSs also play an important role in the pathogenic mechanism of many animal pathogens *Legionella pneumophila*, *Bordetella pertussis*, *Coxiella burnetii*, *Bartonella henselae*, *Brucella spp.* and *Helicobacter pylori* as well as plant pathogen *Agrobacterium tumefaciens* [37, 38]. VirB/D4 T4SS of *S. maltophilia* is related to the well-known plant pathogens of *Xanthomonas* species, a
phylogenetic relative of *S. maltophilia*, which mediates killing of the other bacterial cell by T4SS but not involved in virulence [37]. In the latest study by Nas et al., they suggested that VirB/D4 T4SS in *S. maltophilia* inhibits apoptosis in an epithelial cell to enhance attachment while it promotes apoptosis in infected mammalian macrophages to escape from phagocytosis [36]. The study further revealed that VirB/D4 T4SS in *S. maltophilia* stimulates the growth and mediates inter bacterial killing of other bacteria in the complex microbial community [36]. Thus, by considering the role of VirB/D4 T4SS in virulence, adaptation in the complex microbial community and its upregulation at 37 °C suggests a temperature-dependent strategy for pathoadaptation.

**Upregulation of the genes involved in the aerotaxis, cation diffusion facilitator family transporter and LacI family transcriptional regulators**

Interestingly, increased expression of genes involved in aerotaxis, which is also known as energy taxis at 37 °C. It is a behavioural response that guides bacterial cells to navigate toward micro-environments where oxygen concentration, energy sources and redox potential are optimal for growth [39]. This process is coordinated by aerotaxis receptor Aer, which measures redox potential. It infers energy levels via a flavin adenine dinucleotide (FAD) cofactor bound to a cytoplasmic PAS domain [39, 40]. In *S. maltophilia* ATCC 13637, two genes (DP16_RS09455 and DP16_RS09460) that encode for FAD-binding domain protein and PAS sensor domain-containing protein are transcribed as single transcript and are overexpressed at 37 °C. This may help *S. maltophilia* to adapt and colonize different niches with a different oxygen gradient. Thus, further experiments are required to understand the role of aerotaxis in *S. maltophilia* adaptation to human host and virulence. Reports are citing the role of aerotaxis in an adaptation of *Campylobacter jejuni* at human gut with different oxygen gradient and in *Ralstonia solanacearum* it is required for the biofilm formation [41, 42]. The role of the aerotaxis in virulence of bacteria is not fully understood, but it plays an important role in the adaptation of bacterium toward its host [43].

Among the upregulated gene, DP_RS06915, that code for cation diffusion facilitator (CDF) family transporter is important for the transition of metals efflux from the cytosol to periplasm. CDF transporter plays a role in the transition metal tolerance, i.e. exporting metal surplus from cell to avoid excessive accumulation and toxicity. Apart from the role in the transition metals efflux, they also participate in the infection process in *P. aeruginosa* [44]. As the transcription of CDF was increased at 37 °C and by considering its possible role in the infection process, it is necessary to assess the role of CDF in virulence and adaption of *S. maltophilia*.

The transcription regulator of LacI family DP16_RS10420 is overexpressed at 37 °C. This family of transcriptional regulators is known to play an essential role in the carbohydrate uptake or metabolism and virulence [45–47]. Upregulation of the gene *fruR*, which is a transcription factor and belongs to the LacI family was observed at 37 °C, suggesting it may play an important role in adaptation and virulence. Therefore, future studies are needed to reveal the role of these genes in infection and adaptation to human body temperature.

**Human body temperature is not heat stress for *S. maltophilia***

The variation of temperature is considered as one of the important stress factors that induces bacterial heat-shock response to adapt and survive thermal stress conditions. Previous studies have reported the heat-induced changes in *S. maltophilia* including changes in the expression of various heat-shock proteins at higher temperature (37 °C) [48]. In our transcriptome study, we observed a significant downregulation of *cspa2* gene that encodes for cold-shock protein at 37 °C suggesting its role in adaptation to lower environmental temperature. Despite the presence of heat-shock chaperons in *S. maltophilia*, we did not find differential gene expression of heat-shock response genes, which is generally indicative of heat stress. This suggests that *S. maltophilia* has evolved to thrive at human body temperature without a need to activate protective surveillance responses against heat stress. Overall, this emphasizes that human body temperature is not heat stress for *S. maltophilia*. This kind of response was also reported in the environmentally originated opportunistic pathogen *Pseudomonas aeruginosa* during growth at 37 °C [2].

In an earlier study, De Carolis and co-workers reported upregulation of GroEL [48]. As the authors checked the expression of GroEL by RT PCR by giving heat shock to cells already growing at 27 °C to high temperature unlike in our case where cells were separately grown at 28 and 37 °C. Further correlation between mRNA and protein expression can always vary due to various factors such as half-lives and post-transcription machinery. Hence the effect of experimental approaches and culture conditions need to be taken into account to understand regulation of genes and its functional relevance.

Temperature is known to alter susceptibility in bacteria. In *Escherichia coli*, cold stress makes it susceptible to glycopeptide antibiotics [49], but in the case of *S. maltophilia*, higher temperature makes it susceptible to aminoglycosides [50]. In our study, the number of downregulated genes is far more than the number of upregulated genes, indicating temperature-dependent modulation of physiology, thereby affecting its success as an opportunistic pathogen. This corroborates with it being relatively less successful than its other non-fermenting Gram-negative bacilli (NFGN) pathogens like *Pseudomonas aeruginosa* and the Bcc complex. However, considering tremendous taxonogenomic diversity reported in the population of *S. maltophilia* [9, 51], it may, in its course of evolution, be the next major nosocomial pathogen.

In addition to clinical, *S. maltophilia* complexes have species from diverse lifestyles. Here, we looked for status of all the 81 pathoadaptive or differentially expressed genes status in all the species of Smc (Fig. 5). While, motility and T4SS genes
in a few of the other species of genus *Stenotrophomonas*. Energy production, metabolism and transcription regulators are largely present in all the species of Smc. Overall phylogenomic-based transcriptomic understanding reveals that the transition and success of *S. maltophilia* species in the genus has been intricate by modulating functions related to immune evasion as seen by downregulation of flagella, protection from host defence responses as seen by downregulation of genes involved in motility apart from other cellular processes related to physiology, replication and transcription (Fig. 6). Further molecular genetic studies on the differentially expressed that are unique to *S. maltophilia* may allow the success of this species to be understood as an opportunistic human pathogen.

**CONCLUSION**

Current work is a high-resolution comprehensive comparative analysis of RNA-Seq based transcriptome of opportunistic pathogen *S. maltophilia*. This study has provided a framework for studying the molecular mechanism underlying transition of an environmental bacterium to become a successful human pathogen. The study also suggests how *S. maltophilia* is a matter of grave concern to the immunocompromised patient. Further, studies on the characterization of differentially expressed genes of *S. maltophilia* at physiological temperature will give more insights into its adaptation to human host and pathogenesis.

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**Author contributions**

P.P.P., S.M., S.K. have prepared the RNA-Seq library preparation and performed transcriptome sequencing on Illumina MiSeq platform. P.P.P., S.K. and K.B. have performed computational analysis of RNA-Seq data analysis. P.P.P. and A.K. have performed motility assay, growth curve and validation of differentially expressed genes by RT-qPCR. A.K., S.K. and P.P.P. performed transmission electron microscopy (TEM). P.P.P. has drafted the manuscript with inputs from S.K., K.B. and A.K. P.B.P. and P.P.P. have conceived and designed the experiments with inputs from S.K., A.K. and K.B.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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