Thermal endurance by a hot-spring-dwelling phylogenetic relative of the mesophilic *Paracoccus*

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ABSTRACT

High temperature growth/survival was revealed in a phylogenetic relative (strain SMMA_5) of the mesophilic *Paracoccus* isolated from the 78-85°C water of a Trans-Himalayan sulfur-borax spring. After 12 h at 50°C, or 45 minutes at 70°C, in mineral salts thiosulfate (MST) medium, SMMA_5 retained ~2% colony-forming units (CFUs), whereas comparator *Paracoccus* had 1.5% and 0% CFU left at 50°C and 70°C respectively. After 12 h at 50°C, the thermally-conditioned sibling SMMA_5_TC exhibited ~1.5 time increase in CFU-count; after 45 minutes at 70°C, SMMA_5_TC had 7% of the initial CFU-count intact. 1000-times diluted Reasoner's 2A medium, and MST supplemented with lithium, boron or glycine-betaine (solutes typical of the SMMA_5 habitat), supported higher CFU-retention/CFU-growth than MST. With or without lithium/boron/glycine-betaine in MST, a higher percentage of cells always remained viable (cytometry data), compared with what percentage remained capable of forming

single colonies (CFU data). SMMA_5, compared with other *Paracoccus*, contained 335 unique genes, mostly for DNA replication/recombination/repair, transcription, secondary metabolites biosynthesis/transport/catabolism, and inorganic ion transport/metabolism. It's also exclusively enriched in cell wall/membrane/envelope biogenesis, and amino acid metabolism, genes. SMMA_5 and SMMA_5_TC mutually possessed 43 nucleotide polymorphisms, of which 18 were in protein-coding genes with 13 nonsynonymous and seven radical amino acid replacements. Such biochemical and biophysical mechanisms could be involved in thermal stress mitigation which streamline the cells' energy and resources towards system-maintenance and macromolecule-stabilization, thereby relinquishing cell-division for cell-viability. Thermal conditioning apparently helped memorize those potential metabolic states which are crucial for cell-system maintenance, while environmental solutes augmented the indigenous stability-conferring mechanisms.

IMPORTANCE

For a holistic understanding of microbial life's high-temperature adaptation it is imperative to explore the biology of the phylogenetic relatives of mesophilic bacteria which get stochastically introduced to geographically and geologically diverse hot spring systems by local geodynamic forces. Here, endurance of high heat, up to the extent of growth under special (habitat-inspired) conditions, was discovered in a hot-spring-dwelling phylogenetic relative of the mesophilic *Paracoccus* species. Thermal conditioning, extreme oligotrophy, metabolic deceleration, presence of inorganic/organic solutes, and typical genomic specializations were found to be the major enablers of this

conditional (acquired) thermophilicity. Feasibility of such phenomena across the taxonomic spectrum can well be paradigm-changing for the established scopes of microbial adaptation to the physicochemical extremes. Applications of conditional thermophilicity in microbial process biotechnology may be far reaching and multi-faceted.

KEYWORDS

Hot spring mesophiles, *Paracoccus*, thermal endurance, thermal conditioning, acquired thermophilicity, genome specialization, oligotrophy, boron, lithium, glycine-betaine

RUNNING TITLE

Thermal endurance by a hot spring mesophile

INTRODUCTION

Association of the typically thermophilic or hyperthermophilic bacteria and archaea with terrestrial hydrothermal habitats is axiomatic. Accordingly, our knowledge on microbial adaptation to high temperature is based largely on hot spring isolates that grow *in vitro* either obligately at ≥80°C (1-2) or facultatively between 30°C and 80°C (3-4). Members of mesophilic microbial groups (taxa having no member reported for laboratory growth at >45°C), on the other hand, though unexpected in high-temperature environments, often get stochastically introduced by local geodynamic forces to the hot spring systems (5-7), where they are detected mostly via sequencing and analysis of metagenomes (6-15), and sometimes as pure culture isolates (12, 16, 17). In such a scenario, for a holistic

understanding of microbial life's high-temperature adaptation, it becomes imperative to explore the biology of the phylogenetic relatives of mesophilic bacteria which happen to be there in geographically and geologically distinct hot spring habitats. That said, most studies on thermotolerance by mesophilic bacteria have thus far been centered on economically or clinically important strains isolated from environments other than hydrothermal ecosystems (18-22). Here we investigate high-temperature growth/survival in relation to the physiology, cell biology, and genomics of a novel, facultatively chemolithoautotrophic strain of the alphaproteobacterial genus *Paracoccus* (named SMMA_5) that was isolated (12) from the vent-water of a Trans-Himalayan sulfur-borax spring called Lotus Pond, located in the Puga geothermal area of eastern Ladakh (India), at an altitude of 4436 m, where water boils at ~85°C (23). Remarkably, whereas no member of *Paracoccus* grows at >45°C *in vitro*, the temperature of the habitat of SMMA_5 ranges between 78°C and 85°C (7, 12, 23).

In order to understand how SMMA_5 responds to thermal stress, increase/decrease in the number of colony forming units (CFUs) present in the culture was tested at different temperatures (37°C-70°C); frequency of viable cells within the culture was determined via fluorescein diacetate (FDA) staining followed by flow cytometry. Potential effects of thermal conditioning on the isolate's ability to grow or survive at high temperatures were tested by subjecting the organism to different iterations of "heat exposure and withdrawal" cycles. Previous geomicrobiological explorations of Lotus Pond had shown that the snow-melts and denuded soil/sediments, which infiltrate the shallow geothermal reservoir of Puga via local tectonic faults, introduce mesophilic bacteria into the hot spring system (7). Since the soil/sediment systems of the frigid deserts of Ladakh are typically poor in organic carbon (24), and

because the vent-water of Lotus Pond has low dissolved solutes concentration (7), the plausible role of oligotrophy in thermal endurance by SMMA_5 was tested via high-temperature incubation in different dilution grades of Reasoner's 2A (R2A) medium, typically used for growing oligotrophic bacteria (25). Dissolved solutes such as ions of boron and lithium, which are typical of the Puga hot springs including Lotus Pond (7, 14, 23), have been hypothesized previously as the *in situ* mitigators of the biomacromolecule-disordering effects of heat, and therefore the facilitators of the colonization of high-temperature sites by mesophilic bacteria (14). Accordingly, sodium tetraborate and lithium hydroxide were tested for their potential effects on the high-temperature growth/survival of SMMA_5. Furthermore, previous culture-independent community analyses of Lotus Pond's vent-water (7, 12) had revealed the presence of several species belonging to glycine-betaine-producing genera such as *Bacillus*, *Ectothiorhodospira*, *Escherichia*, *Halomonas*, *Pseudomonas* and *Staphylococcus* (26-28). Since the osmoprotective compatible solute glycine-betaine is known to mitigate a wide range of physicochemical stressors (29), its potential effect on the high-temperature growth/survival of SMMA_5 was tested alongside boron and lithium. Finally, to know whether thermal endurance had a genetic foundation, the whole genome of SMMA_5 was sequenced and analyzed in conjunction with that of its thermally conditioned variant SMMA_5_TC.

RESULTS

Taxonomic identity and growth characteristics of SMMA_5

The 16S rRNA gene sequence of SMMA_5 exhibited closest (96.2-97.5%) similarities with homologs from diverse *Paracoccus* species; in the phylogenetic tree constructed,

the isolate shared a major clade with *Paracoccus denitrificans*, *Paracoccus kondratievae*, *Paracoccus pantotrophus*, *Paracoccus versutus*, *Paracoccus bengalensis*, and several other species (Fig. S1). In the phylogeny reconstructed based on 92 conserved marker gene sequences (Fig. 1), SMMA_5 clustered in a major clade encompassing *Paracoccus aestuariivivens*, *Paracoccus aminophilus*, *Paracoccus aminovorans*, *P. denitrificans*, *P. kondratievae*, *Paracoccus laeviglucosivorans*, *Paracoccus limosus*, *Paracoccus litorisediminis*, *Paracoccus lutimaris*, *P. pantotrophus*, *Paracoccus sulfuroxidans*, *Paracoccus thiocyanatus*, *P. versutus* and *Paracoccus yeei* (notably, no genome sequence was available for *P. bengalensis*, so this organism could not be included in the phylogenomic analysis).

The SMMA_5 genome exhibited 23.7%, 23.8% and 21.6% DNA-DNA hybridization, *in silico*, with the genomes of the phylogenetically closest species *P. aminovorans*, *P. denitrificans* and *P. kondratievae* respectively (Table S1). Orthologous genes-based average nucleotide identities of SMMA_5 with the above three genomes were 81.2%, 81.4% and 78.8% respectively. These data indicated that SMMA_5 was a potentially novel species of *Paracoccus*.

When SMMA_5 was incubated in mineral salts thiosulfate (MST) medium for 12 h, $>10^4$ times increases in CFU-count were observed at 37°C-45°C; at 50°C, 2% of the initial CFU-count remained in the culture, whereas at 60°C and 70°C no CFU was left (Fig. 2A and B). However, until 45 minutes in MST at 70°C, SMMA_5 had 1.6% of the initial CFU-count remaining in the culture (Fig. 3A and B).

The comparator chemolithoautotroph *P. pantotrophus* LMG 4218, when incubated in MST for 12 h, exhibited $>10^4$ times increase in CFU-count at up to 40°C; at 45°C $\sim 10^3$ times increase in CFU-count was recorded, while at 50°C, 1.5% of the initial

149 CFU-count remained in the culture; at 60°C and 70°C no CFU was left (Fig. 2C and D);
150 no CFU was also there after 45 minutes in MST at 70°C (Fig. 3A and B).
151 *Escherichia coli* K-12, when incubated in mineral salts dextrose (MSD) for 12 h,
152 showed >10^4 times CFU-growth at up to 40°C; whereas only 5.8% CFU remained in the
153 culture at 45°C, no CFU was left at ≥50°C (Fig. 2E and F); no CFU was also there after
154 45 minutes at 70°C (Fig. 3A and B).
155
156 **Thermal conditioning enhances high-temperature growth/survival**
157 For maintenance, SMMA_5 was grown for 12 h at 37°C, and stored at 4°C for 28 days
158 until the next transfer. On the other hand, its thermally conditioned cell-line (sibling
159 strain), designated as SMMA_5_TC, was raised and maintained by first incubating for
160 12 h at 50°C, then reviving growth for 12 h at 37°C, and finally storing at 25°C for 14
161 days until the next transfer involving the same 50°C-37°C incubation procedure (Fig.
162 4A). All data presented for SMMA_5 and SMMA_5_TC were obtained from experiments
163 carried out after 15 and 30 transfer cycles respectively.
164 When tested for growth in MST medium at 37°C, 40°C and 45°C, SMMA_5_TC
165 (Fig. 2G and H) exhibited phenotypes identical to those of SMMA_5 (Fig. 2A and B).
166 However, after 12 h at 50°C in MST, SMMA_5_TC exhibited ~1.5 times increase in the
167 CFU-count of the culture with respect to the 0 h level (Fig. 2G and H), whereas under
168 identical conditions SMMA_5 retained only 2% of the initial CFU-count (Fig. 2A and B).
169 Albeit, SMMA_5_TC (Fig. 2G and H) did not have any CFU left after 12 h in MST at
170 60°C and 70°C, after 45 minutes in MST at 70°C it had 7% of the initial CFU-count intact
171 (Fig. 3A and B).

172 Subsequently, experiments were carried out to know the effects of only a few
173 thermal-conditioning cycles on the growth or CFU-retention by SMMA_5 at 50°C and
174 70°C (each cycle involved incubation for 12 h at 50°C, followed by 12 h at 37°C; Fig.
175 4B). When the six sibling strains generated after one round of thermal conditioning were
176 individually incubated for 12 h at 50°C, 2.5%, 4.3%, 1.7%, 3.7%, 5.8% and 2.9% (on an
177 average 3.5%) of their initial CFU-counts were retained (Fig. 5A and B). Likewise, when
178 these six sibling strains were individually incubated for 45 minutes at 70°C, 3.8%, 1.8%,
179 4.2%, 3.2%, 2.7% and 4.0% (on an average 3.3%) of their initial CFU-counts were
180 retained (Fig. 5E and F).
181 When the six sibling strains generated after five rounds of thermal conditioning
182 were individually incubated for 12 h at 50°C, 23.7%, 16.1%, 11.4%, 18.8%, 11.0% and
183 8.9% (on an average 15%) of their initial CFU-counts were retained (Fig. 5C and D).
184 Furthermore, when these six sibling strains were individually incubated for 45 minutes at
185 70°C, 10.5%, 3.1%, 4.0%, 2.2%, 2.6% and 15.2% (on an average 6.2%) of their initial
186 CFU-counts were retained (Fig. 5G and H).
187
188 **At high temperatures, cell viability is more than divisibility**
189 After 12 h at 50°C, as well as after 45 minutes at 70°C, the percentages of SMMA_5 and
190 SMMA_5_TC cells remaining viable or metabolically active were far higher than the
191 percentages of cells remaining ready to divide and form single colonies (CFU-count
192 data). As a mark of their metabolically active state, cell populations were tested post
193 high-temperature incubation for their ability to uptake FDA and hydrolyze it to
194 fluorescein, which in turn was detected via flow cytometry. In that way, after 12 h
195 incubation at 50°C in MST, 98.7% and 99.56% cells of SMMA_5 and SMMA_5_TC were

196 found to remain viable respectively (Fig. 3C and D); similarly, 23.26% and 51.43% cells
197 of SMMA_5 and SMMA_5_TC remained viable after 45 minutes at 70°C in MST (Fig. 3E
198 and F).
199
200 **Environmental solutes enhance thermal endurance or elicit moderate**
201 **thermophilicity**
202 When SMMA_5 was incubated at 50°C for 12 h in MSTB, MSTL and MSTG, i.e. MST
203 supplemented with 16 mM boron, 1 mM lithium and 10 mM glycine-betaine respectively,
204 14.4%, 29.2% and 224% of the initial CFU-counts were present in the respective
205 cultures (Fig. 6C and S2C). For SMMA_5_TC, 12 h incubation at 50°C in MSTB, MSTL
206 and MSTG led to the CFU-growths equivalent to 170%, 230% and 370% of the initial
207 counts respectively (Fig. 6D and S2D). Notably, SMMA_5 and SMMA_5_TC were also
208 found to release 8-10 mM glycine-betaine in the medium after 12 h incubation in MST at
209 50°C.
210 After 45 minutes at 70°C in MSTB, MSTL or MSTG, SMMA_5 retained 2.9%,
211 21.4% and 5.5% of the initial CFU-count respectively (Fig. 6E and S2E). For
212 SMMA_5_TC, 45 minute incubation at 70°C in MSTB, MSTL and MSTG led to the
213 retention of 10.4%, 29.2% and 15.8% of the initial CFU-count respectively (Fig. 6F and
214 S2F). Furthermore, 37°C incubation of either strain for 12 h in MSTB, MSTL and MSTG
215 showed that boron, lithium and glycine-betaine were not only non-toxic to them but also
216 considerably stimulatory to their growth at this temperature (Fig. 6A and B; Fig. S2A and
217 B).
218 The three environmental solutes also exhibited pronounced influence on the
219 viability of SMMA_5 and SMMA_5_TC cells at 70°C (notably, at 50°C, almost 100% cell

220 viability was already recorded for both strains, without any environmental solute in the
221 medium). After incubation in MSTB, MSTL and MSTG for 45 minutes at 70°C, 43.45%,
222 71.36% and 55.17% cells of SMMA_5 were found to remain viable respectively (Fig. 7A-
223 C). Likewise, after 45 minutes in MSTB, MSTL and MSTG at 70°C, 66.04%, 89.31% and
224 76.35% cells of SMMA_5_TC were found to remain viable respectively (Fig. 7D to F).
225

226 **Oligotrophy helps endure high temperature**

227 When incubated in R2A for 12 h at 37°C, both SMMA_5 and SMMA_5_TC exhibited
228 $>10^4$ times increase in CFU-count (Fig. 8A and B; Fig. S3A and B); comparable growth
229 was recorded for the two strains in Luria broth after 12 h at 37°C (data not shown). After
230 12 h at 50°C in R2A, SMMA_5 and SMMA_5_TC retained 2% and 2.5% of the initial
231 CFU-counts respectively (Fig. 8C and D; Fig. S3C and D), but after 12 h at 50°C in Luria
232 broth, neither strain retained any CFU. Likewise, after 45 minutes at 70°C in R2A,
233 SMMA_5 and SMMA_5_TC retained 0.5% and 1.0% of the initial CFU-counts
234 respectively (Fig. 8E and F; Fig. S3E and F), but their Luria broth cultures retained no
235 CFU after 45 minutes at 70°C.

236 At 37°C, 12 h incubation in 0.1X, 0.01X and 0.001X R2A resulted in progressively
237 lower cellular growth yields, as compared to what was recorded in R2A (1X), for both
238 SMMA_5 and SMMA_5_TC (Fig. 8A and B; Fig. S3A and B).

239 After 12 h incubation at 50°C in 0.1X, 0.01X and 0.001X R2A, SMMA_5 retained
240 2.7%, 15.6% and 33.3% of the initial CFU-count respectively (Fig. 8C and S3C),
241 whereas SMMA_5_TC retained 3.5%, 20.0% and 53.8% of the initial CFU-count
242 respectively (Fig. 8D and S3D).

243 After 45 minutes at 70°C in 0.1X, 0.01X and 0.001X R2A, SMMA_5 cultures
244 retained 2.6%, 9.4% and 14.9% of the initial CFU-count respectively (Fig. 8E and S3E),
245 whereas SMMA_5_TC retained 3.1%, 13.4% and 28.5% of the initial CFU-count
246 respectively (Fig. 8F and S3F).
247
248 **pH 7.5 is optimum for thermal endurance**
249 The optimum pH for growth or CFU-retention at 37°C was tested in R2A, while the same
250 at 50°C and 70°C was tested in 0.001X R2A. The media types were chosen based on
251 their best support for growth or CFU-retention at the respective temperatures (Fig. 8);
252 pH optima were not determined in MST medium because its salt ingredients precipitate
253 out at pH >7.0 and temperature ≥50°C. For both SMMA_5 and SMMA_5_TC, R2A
254 having pH 7.5 supported maximum growth after 12 h at 37°C (Fig. S4A and B; Fig. S5A
255 and B); likewise, 0.001X R2A having pH 7.5 supported maximum CFU-retention after 12
256 h at 50°C (Fig. S4C and D; Fig. S5C and D), as well as 45 minutes at 70°C (Fig. S4E
257 and F; Fig. S5E and F).
258
259 **Rapid growth is not sustainable amid high heat**
260 SMMA_5_TC, when grown at 70°C in 0.001X R2A having an initial pH 7.5 and fortified
261 simultaneously with 4 mM $Na_2B_4O_7.10H_2O$, 1 mM $LiOH.H_2O$ and 10 mM $C_5H_{11}NO_2$,
262 exhibited a 1.4 time increase in CFU-count (from 1.9×10^6 mL^{-1} to 2.73×10^6 mL^{-1}, as
263 averages of three different experiments where standard deviations of the data were <2%
264 of the means) after 45 minutes of incubation; but after 1 h, no CFU (or for that matter
265 FDA-stained cell) was left in the culture.
266

Genomic uniqueness of the hot spring *Paracoccus*

The 3.09 Mb and 3.06 Mb draft genomes of SMMA_5 and SMMA_5_TC, as represented in 283 and 247 contigs (all >200 bp long), had G+C contents of 65.78% and 65.83% respectively (Table S1). SMMA_5 contained 3,234 potential open reading frames (ORFs) or coding sequences (CDSs), out of which 2498 and 503 encoded known and unknown (hypothetical) proteins respectively; 16S, 23S, and 5S rRNA genes were identified together with 49 tRNA genes. The completeness level of this genome was estimated to be 98.23% (Table S1), based on the presence of 556, out of the total 568, *Paracoccus*-specific conserved marker genes curated in CheckM database. The draft genome of SMMA_5_TC, on the other hand, was found to include 3,163 potential CDSs, out of which 2511 and 477 encoded known and unknown proteins respectively; 16S, 23S, and 5S rRNA genes were identified alongside 47 tRNA genes. Completeness of the SMMA_5_TC genome was 98.41% (Table S1), based on the presence of 554 *Paracoccus*-specific marker genes. The average isoelectric point (pI) of all the putative proteins encoded by SMMA_5 and SMMA_5_TC were predicted as 6.86 and 6.87 respectively; corresponding values for all other *Paracoccus* species except *Paracoccus contaminans* were lower (Table S2; Fig. S6).

Pan genome analysis (PGA) involving SMMA_5, and 44 other *Paracoccus* species having near-complete genome sequences available in the GenBank (Table S1), revealed 822 core genes for the genus. While SMMA_5 had 1742 accessory, 335 unique and 30 exclusively-absent genes, the corresponding numbers in the comparators ranged between 1528-3451, 159-817 and 0-45 (Table S3). Of the 335 unique genes of SMMA_5, 186 encoded hypothetical proteins, whereas 149 coded for putative functional proteins, of which, again, only 83 were ascribable to one or more clusters of orthologous

291 group (COG) categories: predominantly, DNA replication, recombination and repair;
292 transcription; secondary metabolites biosynthesis, transport and catabolism; inorganic
293 ion transport and metabolism; and cell wall/membrane/envelope biogenesis (Table S4).
294 The metabolic potentials of SMMA_5 were compared with those of the
295 phylogenomically closest *Paracoccus* species (see Fig. 1) on the basis of their gene-
296 content under different COG categories. Two-way clustering of the genomes analyzed
297 and COG categories identified revealed close functional relationships of SMMA_5 with
298 *P. dinitrificans* and *Paracoccus thiocyanatus* on one hand, and *P. aminophilus* and
299 *Paracoccus sulfurioxidans* on the other (Fig. 9A). Notably, in the phylogenomic tree, the
300 first two species were closely related to SMMA_5, but the second pair diverged widely
301 (Fig. 1). *P. pantotrophus P. yeei*, *P. kondratievae*, *P. aminovorans* and *P. lutimaris*
302 showed the next closest relationships with SMMA_5 in the two-way dendogram (Fig.
303 9A), but out of these five species only the first four shared the same branch with
304 SMMA_5 in the phylogenomic tree whereas *P. lutimaris* was in a completely distinct
305 branch (Fig. 1). *P. versutus*, which was phylogenomically closely related to SMMA_5
306 (Fig. 1), diverged widely from the new isolate in terms of gene-content under different
307 COG categories (Fig. 9A). Statistically analysis further revealed the two COG categories
308 (i) cell wall/membrane/envelope biogenesis, and (ii) amino acid transport and
309 metabolism, to be exclusively enriched in SMMA_5; lipid transport and metabolism was
310 found to be enriched in SMMA_5 and *P. aminovorans* (Fig. 9B).
311
312 **Genomic aspects potentially linked to thermal adaptation**
313 The genomes of both SMMA_5 and SMMA_5_TC encompassed a large repertoire of
314 heat shock proteins and molecular chaperones. These included (i) one copy each of the

315 co-chaperone GroES, the heat shock protein HspQ, the heat-inducible transcriptional
316 repressor HrcA, the Hsp33 family molecular chaperone HslO, the co-chaperone GrpE,
317 and the RNA polymerase sigma 32 factor RpoH; (ii) two copies each of the genes
318 encoding the chaperonin GroEL, Hsp20 family protein, and the Hsp70 family protein
319 DnaK; and (iii) three copies of the molecular chaperone DnaJ (Hsp40). Genes for heat
320 shock protein Hsp90 and RNA polymerase sigma E factor, which are typically
321 associated with heat stress management in *E. coli*, were not found in the hot spring
322 *Paracoccus* genomes (Table S5). On the other hand, the presence of genes for the
323 redox-regulated, Hsp33-family chaperone HslO – which is present in most thermophilic
324 bacteria to protect thermally/oxidatively damaged proteins from irreversible aggregation
325 – distinguished the genomes of SMMA_5 and SMMA_5_TC from those of *E. coli* and *P.*
326 *denitrificans*. Furthermore, the genomes of SMMA_5 and SMMA_5_TC encoded several
327 other proteins that can be potentially decisive in thermal adaptation by means of their
328 regulated expression (Table S6).
329 The draft genomes of SMMA_5 and SMMA_5_TC showed 99.9748% sequence
330 similarity over a total alignment length of 3061077 nucleotides (unaligned portions of the
331 two genomes were attributable to their incomplete sequencing, rather than the presence
332 of unique genes). Of the total 43 instances of single nucleotide polymorphism (SNP)
333 detected across the alignment, 18 were in protein-coding genes, 18 were in frame-
334 shifted pseudogenes, whereas 7 were in non-coding regions (Table S7). Out of the 18
335 nucleotide substitutions detected in protein-coding genes, 13 were nonsynonymous (ns)
336 SNPs while five were synonymous (silent) mutations (sSNPs). Of the 13 nsSNPs, again,
337 nine and four involved transitions and transversions respectively, while seven involved
338 such radical replacements of amino acids (e.g., polar to non-polar, or aromatic-group-

339 containing to alkyl-group-containing, amino acids, or vice versa) that may lead to
340 changes in the corresponding protein structure and function (Table S7).
341
342 **DISCUSSIONS**
343 A complex set of geobiological and biophysical factors worked in conjunction with
344 specialized genomic attributes to confer thermal endurance, or even moderate
345 thermophilicity, to the hot-spring-dwelling relative of mesophilic *Paracoccus*. While the
346 thermally unconditioned isolate SMMA_5 had an intrinsically higher frequency of CFU-
347 retention at 50°C and 70°C, compared with other *Paracoccus* (and *E. coli*), its sibling
348 SMMA_5_TC, which had been thermally conditioned for more than 30 transfer cycles,
349 showed either growth or yet higher frequencies of CFU-retention at these temperatures.
350 Corroboratively, thermal conditioning for only five transfer cycles brought about, on an
351 average, eight and four times increases in the CFU-retention frequencies of SMMA_5 at
352 50°C and 70°C respectively. Geochemical and microbial solutes further improved the
353 growth/survival efficacies at 50°C and 70°C, as did extreme oligotrophy at pH 7.5.
354 Overall it seems that during their residence in the solute-poor, circum-neutral pH, and
355 near-boiling water of Lotus Pond (7, 12), cell populations of this novel *Paracoccus* had
356 already acquired such heritable structural and functional attributes which are conducive
357 for the mitigation of high heat. *Ex situ*, the potential cellular memories conferring heat
358 endurance (or moderate thermophilicity) were on the wane in the thermally
359 unconditioned culture (SMMA_5), while the same were retained relatively better in the
360 thermally conditioned variant (SMMA_5_TC).
361

362 **Prior experience of high temperature enhances heat endurance or confers**
363 **moderate thermophilicity**
364 Pre-exposure to sub-lethal stress conditions is known to enhance a microorganism's
365 resilience to higher levels of stress (30). Bacteria are endowed with diverse memory
366 mechanisms, including those which involve the inheritance and propagation of
367 epigenetic states associated with adaptive advantages (31, 32). Of the various
368 metabolic systems that are known to involve heritable epigenetic memory, ion-channel-
369 mediated signaling and ion flux machineries (33-35) appear to be pertinent to thermal
370 endurance in view of the observed influence of environmental solutes on growth and
371 survival at high temperatures.
372 Under almost all the culture conditions tested, the standard deviations of the CFU-
373 retention data for SMMA_5_TC were higher than those for SMMA_5. Concurrent to this
374 trend, the sibling strains generated via one or five cycles of thermal conditioning of
375 SMMA_5, did not show mutually equal frequencies of CFU-retention at any of the high-
376 temperatures tested. These data collectively indicated that in the hot-spring-dwelling
377 *Paracoccus*, thermal conditioning via potential trait memorization could be a
378 multifactorial, and asymmetrically inherited (32), process that involves a "bet and
379 hedging" strategy, where heterogeneous populations of phenotypically-distinct but
380 genotypically-identical cells are formed and maintained by a bacterium to ensure that
381 one sub-population or the other can always withstand, and adapt to, unknown
382 environmental challenges of the future (36).
383
384
385

386 **Role of environmental solutes in enhancing thermal endurance or eliciting**
387 **moderate thermophilicity**
388 Out of the three environmental solutes tested, the alkali metal lithium was most effective
389 in increasing the frequency of CFU-retention, as well as the percentage of viable cells,
390 at 70°C, for both SMMA_5 and SMMA_5_TC, as compared to their respective MST
391 phenotypes. At 50°C too, lithium fortification of MST increased the frequency of CFU-
392 retention and CFU-growth for SMMA_5 and SMMA_5_TC respectively. These
393 phenotypes were consistent with previous observations where *Listeria monocytogenes*
394 exposed to 62.8°C for 10-20 minutes was recovered after 48-144 h, by adding 7 g L^{-1}
395 LiCl to the 30°C revival cultures (18). Thermoprotective effect of lithium could be linked,
396 but not necessarily restricted, to its activities at the periphery of cells. Li^+ efficiently forms
397 hydrogen bonds with nearby water molecules, as well as charged surfaces such as cell
398 membranes, thereby modifying their intrinsic van der Waals, and electrostatic, forces
399 (37). Li^+ also competes for ligand-binding sites of biomacromolecules that are otherwise
400 reserved for Na^+ or Mg^{2+} (38). By virtue of these biophysical maneuvers Li^+ can not only
401 stabilize biomacromolecules but also enhance the permeability of those constituting
402 cellular membranes, which in turn can enhance the entry of other thermo-/osmo-
403 protective solutes (39, 40).
404 The compatible solute glycine-betaine also significantly increased the frequency of
405 CFU-retention, as well as the percentage of viable cells, at 70°C, for both SMMA_5 and
406 SMMA_5_TC. Furthermore, at 50°C, glycine-betaine fortification of MST brought about
407 an unprecedented CFU-growth for SMMA_5, and a small but definite increase in the
408 existing CFU-growth of SMMA_5_TC. Glycine-betaine is taken-up/synthesized in large
409 amounts by halophilic/halotolerant microbes as their secondary response to high

410 external salt concentrations (28). However, besides acting as osmotic balancers,
411 compatible solutes can also impart a general stabilizing effect on biomacromolecules
412 (41, 42). As the highly-soluble glycine-betaine molecules accumulate within the cell,
413 their preferential exclusion from the immediate hydration sphere of proteins leads to a
414 non-homogeneous distribution within the cell-water, thereby causing a thermodynamic
415 disequilibrium (43). This disequilibrium is minimized via reduction in the volume of cell-
416 water from which the solute is excluded; this, in turn, is achieved via reduction in the
417 surface-area or volume of the proteins through increases in sub-unit assembly and
418 stabilization of secondarily-folded tertiary structures (43). Such biophysical maneuvers
419 at the molecular level can add-up at the cellular level to confer tolerance against any
420 physicochemical stressor that tends to disrupt the system by increasing the disorder
421 within biomacromolecules (43, 44).

422 Supplementing MST with boron also led to a small but definite increase in the
423 frequency of CFU-retention, as well as the percentage of viable cells, at 70°C, for both
424 SMMA_5 and SMMA_5_TC. At 50°C, boron addition to MST marginally increased the
425 frequency of CFU-retention and CFU-growth for SMMA_5 and SMMA_5_TC
426 respectively. Boron possesses unique bonding properties that can expand the
427 biophysical functions of macromolecules including their modes of Lewis acidity. *In vitro*
428 insertion of boron to proteins in the form of boronoalanine has been shown to allow
429 dative bond-mediated and site-dependent protein Lewis acid–base-pairing, which in turn
430 can generate new functions, including stability in the face of high temperature or
431 proteolytic attack (45). Furthermore, it is theoretically not impossible that boron bonds
432 with membrane fatty acids to form organoborane complexes which in turn provide cross-
433 linkages, and thereby stability, for structures such as cellular membranes.

434 **Oligotrophy as a strategy for survival at high temperature**

435 After 12 h incubation at 37°C, cellular growth yield of both SMMA_5 and SMMA_5_TC
436 was higher in 1X R2A than in any of the dilution grades of the medium. But, after 12 h
437 incubation at 50°C, as well as after 45 minutes at 70°C, all the dilution grades of R2A
438 supported the retention of higher CFU percentages than undiluted R2A, for both
439 SMMA_5 and SMMA_5_TC. When compared with the corresponding MST phenotype,
440 CFU-retention in the different dilution grades of R2A was

441 - higher for SMMA_5, but not SMMA_5_TC, when incubation was carried out for 12 h
442 at 50°C, and
443 - higher for both SMMA_5 and SMMA_5_TC when incubation was carried out for 45
444 minutes at 70°C.

445 Thus, SMMA_5 and SMMA_5_TC were found to be best adapted to cope 50°C
446 temperature under oligotrophic and chemolithoautotrophic conditions respectively,
447 whereas both strains were best adapted to cope 70°C under oligotrophic condition.
448 These findings, in concurrence with the different effects exhibited by the environmental
449 solutes on thermal endurance, indicated that the hot spring *Paracoccus* potentially
450 modulates its metabolic response to thermal stress with increasing temperature, and
451 such response regulation potentially involves a differential control of the global
452 transcriptome, orchestrated over prolonged thermal conditioning. Overall, the survival
453 strategy amid high heat appears to be aimed at slowing down growth, and prioritizing
454 cell-system maintenance over proliferation.

455 Oligotrophy also affords collateral advantages in combating a wide variety of
456 stresses including high heat (46). It entails a constant ability to take up whatever little
457 nutrients are available in the cells' chemical milieu by using low-specificity, high-affinity,

458 and low-activation-energy, transport systems (47). However, constant nutrient uptake
459 via low-specificity transport systems implies that hydrogen peroxide (H_2O_2) and other
460 reactive oxygen species (ROS) abundant in hydrothermal environments also get
461 unrestricted entry into the cell, as do the potentially thermoprotective inorganic solutes
462 and small organic molecules. Furthermore, cells develop intrinsic resistance against
463 H_2O_2/ROS toxicity via enhanced expression of genes responsible for membrane
464 detoxification, and protein repair and maintenance (48), which in turn protect the cells'
465 macromolecules against the destabilizing effects of heat.
466

467 **Genomic underpinnings of thermal endurance**

468 The hot spring *Paracoccus* possessed 335 unique genes, plus a large repertoire of
469 genes involved in protein/DNA quality management under stress conditions. While many
470 of its genes have potential roles in thermal adaptation, the genome as a whole is
471 enriched in genes for cell wall/membrane/envelope biogenesis, and transport and
472 metabolism of amino acids and lipids. Thermal conditioning, or the lack of it, appears to
473 have a small but definite effect on the genome. This is consistent with the fact that
474 temperature influences the rate and nature of mutations, and thereby natural selection,
475 evolution, and biogeography of a microorganism (49). Of the total 43 SNPs identified
476 across the aligned portions of the SMMA_5_TC and SMMA_5 genomes, 18 were in
477 protein-coding genes. Out of these 18, again, 13 and 5 were nsSNPs and sSNPs
478 respectively: a ratio that is potentially indicative of adaptive evolution (50) or relaxed
479 selective constraints (51) in the thermally conditioned (SMMA_5_TC) lineage. Of the
480 13 nsSNPs detected in protein coding genes, nine and four involved transition and
481 transversion mutations respectively, while seven resulted in radical amino acid

482 replacements having potentials to change protein structures and functions in such ways
483 that can impact activities/phenotypes (Table S7). While radical amino acid
484 replacements often turn out to be beneficial mutations that enhance protein functions
485 (52), those encountered in the putative selenide, water dikinase protein SelD, sulfate
486 adenylyltransferase subunit 2 CysD, and DNA polymerase III subunit gamma/tau,
487 appear to hold major implications for the enhanced thermal endurance or moderate
488 thermophilicity of SMMA_5_TC.

489 Bacteria have a large repertoire of selenocysteine-containing proteins, and both
490 SelD and CysD play vital roles in their biosynthesis. While the role of selenoproteins in
491 cellular stress management is well documented (53), CysD is also involved in the first
492 step of sulfite formation during the assimilatory reduction of sulfate to sulfide (54). Since
493 ROS is a major driver of cell death during thermal stress (55, 56), the highly reduced
494 sulfide species produced during sulfate reduction can potentially defend microbial cells
495 amid high heat (57). The efficiency of these processes may well be enhanced by
496 potentially modified homologs of SelD and CysD.

497 Gamma and tau subunits of DNA polymerase III, the stoichiometric components of
498 the replicative complex, are engaged in loading the processivity clamp beta (58, 59). As
499 hyperthermia inhibits DNA replication by either slowing down or arresting the replication
500 forks depending on the temperature level and cell type (60), it is not unlikely that the
501 DNA polymerase III subunit gamma/tau of SMMA_5_TC has potential structural
502 advantages for high-temperature functioning.

503

504

505 **The putative proteome of hot spring *Paracoccus* is biophysically adapted to the**
506 **environment**

507 With the elevation of temperature and/or increase in the time of heat exposure,
508 conformational changes in the structure of proteins decrease their solubility and tend to
509 precipitate them out of the solution. Solubility of most proteins increase up to the 40-
510 50°C temperature range, and start decreasing irreversibly beyond these levels,
511 depending on the pH of the medium, and also its ionic strength (61). In this scenario,
512 higher electrostatic repulsion between the molecules of a protein, compared with the
513 degree of hydrophobic interactions between them, can promote solubility via enhanced
514 interaction with the solvent. At pH values above their pI, net charge of proteins become
515 negative, so repulsion between their molecules, and thereby their solubility (interactions
516 with water), increases (61). The average pI values predicted for all the putative proteins
517 of SMMA_5 and SMMA_5_TC were 6.86 and 6.87 respectively, with 61.2% and 61.1%
518 of all proteins of the two strains having pI values <6.86 and <6.87 respectively.
519 Furthermore, in oligotrophic media, pH 7.5 was found to be optimum for growth at 37°C,
520 and CFU-retention at 50°C and 70°C, concurrent to which, 65.8% and 65.7% of all
521 SMMA_5 and SMMA_5_TC proteins were predicted to have pI <7.5 respectively. In this
522 context it is further noteworthy that the measured pH of Lotus Pond's vent-water from
523 where SMMA_5 was isolated ranged between 7.2 and 8.0, corresponding to *in situ*
524 temperatures 85°C and 78°C (7). In this way, both *in vitro* pH optimum and the *in situ* pH
525 of the habitat were above the average pI predicted for the total proteomes of SMMA_5
526 and SMMA_5_TC. Moreover, these *in vitro* or *in situ* pH values, at high temperatures,
527 represent considerably alkaline environments, which in the light of the above biophysical

528 considerations appear to be favorable for the conformational integrity, and the solubility,
529 of the majority of proteins of this microorganism.
530
531 **Metabolic deceleration as central to thermal endurance**
532 MST incubation at 50°C and 70°C, with or without lithium, boron or glycine-betaine,
533 showed that a greater proportion of cells always remain metabolically active and viable,
534 compared to what percentage of cells remain ready to divide and form colonies upon
535 withdrawal of heat. Corroboratively, though one or two generations of CFU-growth was
536 achieved over a period of 12 h at 50°C in MST, rapid cell division at a rate lower than
537 the strain's normal generation time (in MST at 37°C, generation time for both SMMA_5
538 and SMMA_5_TC was ~90 minutes) was deleterious to the survival of the population at
539 70°C. All these were reflective of the fact that an overall deceleration of metabolism,
540 underplaying cell division and aimed at achieving a cost-effective maintenance of the
541 cell-system, was central to the thermal endurance strategy of the hot spring *Paracoccus*.
542 Development of "viable but not readily culturable" states in high proportions under
543 different stress conditions is a widespread phenomenon in bacteria (62, 63). At the level
544 of the genome, metabolic deceleration could involve intricate regulation of loci attributed
545 to growth and cell division, oxidative stress management, quality control of
546 biomacromolecules, maintenance of cell wall/envelope integrity, membrane
547 permeability, transport, and energy production/utilization. Concomitantly, thermal
548 conditioning, whether *in situ* or *in vitro*, apparently helps memorize the regulatory states
549 of global gene expression which are central to metabolic slow-down and cell-system
550 maintenance. Eventually, at the level of the biomacromolecules, mitigation of the
551 disordering effects of heat requires such intrinsic entropy-minimizing mechanisms (1, 64)

552 which can accomplish a fine balance between structural integrity (needed to avoid
553 denaturation) and flexibility (necessary to preserve functionality): while the hot spring
554 *Paracoccus* ought to possess native biophysical contrivances to stabilize its
555 macromolecular structures amid high heat (and thereby lower the energy cost of cell-
556 system maintenance), environmental solutes, on their part, appear to augment the
557 indigenous stability-conferring mechanisms. Future studies of transcriptomics,
558 proteomics, and metabolomics, resolved along the dimensions of time, temperature and
559 biogeochemical variables are needed to fully conclude how hot spring mesophiles
560 survive high temperatures over time and space.
561

562 **MATERIALS AND METHODS**

563 **Bacterial strains, media and culture conditions**

564 The facultative chemolithoautotroph SMMA_5 was isolated, as described previously, via
565 enrichment in MST medium that essentially contained a modified basal and mineral salts
566 (MS) solution supplemented with thiosulfate (7, 12). The strain was deposited to the
567 Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India with the
568 public accession number MTCC 12601. Since its isolation SMMA_5 was maintained in
569 R2A medium by growing the culture at 37°C and storing at 4°C, with a standard transfer
570 interval of 28 days. From the main culture, the thermally conditioned variant
571 SMMA_5_TC was created and maintained in the following way. A freshly inoculated
572 R2A broth culture of SMMA_5 was first incubated at 50°C for 12 h and then shifted to
573 37°C for another 12 h. While no turbidity appeared in the culture flask over the 50°C
574 incubation phase, growth corresponding to an OD_{600} of ~0.6 was obtained at the end of
575 12 h at 37°C. The culture flask was stored at 25°C and transferred following the above

procedure after an interval of 14 days (Fig. 4A). The comparator strains *P. pantotrophus* LMG 4218 and *E. coli* K-12 were maintained in Luria Broth by growing the cultures at 37°C and storing at 4°C, with a standard transfer interval of 28 days.

Phylogenetic identification based on 16S rRNA or 92 conserved marker gene sequences

The 16S rRNA gene of the new isolate SMMA_5 was PCR-amplified and sequenced (GenBank accession number LN869532) using the *Bacteria*-specific universal primers 27f and 1492r (65). Pair-wise evolutionary distances between SMMA_5 and other *Paracoccus* species were determined based on their aligned 16S rRNA gene sequences. Subsequent to this, a neighbor-joining tree was constructed following majority rule and strict consensus out of 1000 phylogenetic trees, using the software package MEGAX (66). To check the robustness of the tree's branching pattern, bootstrap values were calculated based on 1000 replicates.

After the *de novo* sequencing and assembly of their genomes (methods given below), the phylogeny of SMMA_5 and SMMA_5_TC was reconstructed based on 92 conserved marker genes, in relation to the other species of *Paracoccus* for which whole genome sequence were available in the GenBank database (a total of 44 non-redundant genomes for different *Paracoccus* species were included in the analysis on a "one genome per species" basis; two more genomes belonging to *Paracoccus* species were also there in the database but they were not considered due to their low levels of completeness; Table S1). Majority of the genes considered for phylogeny reconstruction (i.e., 67 out of 92), belonged to that functional category for clusters of orthologous groups (COGs) which involves translation, including ribosome structure and biogenesis.

600 The up-to-date bacterial core gene (UBCG) set was used to identify and align these
601 marker genes (67). Final tree construction from the aligned marker genes was created
602 using RAxML version 8 (68). Final visualization of the resultant tree was done using
603 interactive Tree of Life (iTOL) version 4 (69).
604
605 **Growth experiments**
606 Growth or decline in the CFU-count of *Paracoccus* cultures at different temperatures
607 (37-70°C) was first tested in MST medium (pH 7.0) containing 20 mM thiosulfate as the
608 sole source of energy and electrons (70). Subsequently, SMMA_5 and SMMA_5_TC
609 were tested at different temperatures in Luria broth, and then in different concentrations
610 of R2A (diluted up to 1000 times, thereby affording an extremely low absolute
611 concentration of organic carbon) that in turn were adjusted to different levels of pH (7.0-
612 9.0). Corresponding experiments for *E. coli* K-12 were carried out in MS supplemented
613 with 4 g L^{-1} dextrose [MSD, pH 7.0 (70)]. Growth or decline in the CFU-count of
614 SMMA_5 and SMMA_5_TC at different temperatures was further tested in MST
615 supplemented with 4 mM $Na_2B_4O_7.10H_2O$ (i.e., 16 mM B), 1 mM $LiOH.H_2O$ (i.e., 1 mM
616 Li) and/or 10 mM glycine-betaine or N,N,N trimethylglycine ($C_5H_{11}NO_2$); the three media
617 types were referred to as MSTB, MSTL and MSTG respectively. Notably, the boron and
618 lithium concentrations used in the culture media were close to those recorded in the
619 habitat of SMMA_5 (7, 14).
620 To test the growth or decline in the CFU-count of a given strain under a particular
621 condition, a seed culture was first prepared in the strain's designated maintenance
622 medium. Inoculum from the log phase seed culture was transferred to the experimental
623 broth, and incubated at the temperature specified for the experiment. To determine the

624 number of CFUs present mL^{-1} of an experimental culture at a given time-point of
625 incubation (including the 0 h), its various dilution grades [created using 0.9% (w/v) NaCl]
626 were plated in triplicates on to R2A (for SMMA_5 and SMMA_5_TC) or Luria (for LMG
627 4218 and K-12) agar, and single colonies counted in each of them after 36 h incubation
628 at 37°C. Colony-counts in the different dilution-plates were multiplied by their respective
629 dilution factors, then summed-up across the plates, and finally averaged to get the
630 number of CFUs that were present mL^{-1} of the experimental culture. Cellular growth
631 yield after a given time-period of incubation was expressed as what percentage of the 0
632 h CFU-count was present in the culture after that period of incubation.
633
634 **Testing the effect of thermal-conditioning cycles on growth or CFU-retention**
635 SMMA_5 was subjected to one or five thermal-conditioning cycles (Fig. 4B), both of
636 which started with 1% inoculum transfer to fresh R2A broth, but subsequently involved
637 one or five iteration(s) of the following incubation cycle respectively: 12 h at 50°C and
638 then 12 h at 37°C. In the case of five iterations, two consecutive incubation cycles were
639 separated by an interval of 48 h at 25°C. In either case (whether thermal-conditioning
640 cycles were one or five), cells from the last 37°C-grown culture were dilution plated on
641 R2A agar, and six discrete colonies were selected as six individual sibling strains of
642 SMMA_5. Finally, each member of the two strain-sets - designated as C1_S1 to C1_S6
643 (Fig. 5A, B, E and F) and C5_S1 to C5_S6 (Fig. 5C, D, G and H), according as they
644 originated from one or five conditioning cycles - was tested individually for its CFU-
645 growth/decline at different temperatures following the procedure already described
646 above.
647

648 **Determining the percentage of viable cells by flow cytometry**

649 CFU-count gave the estimate of what percentage of cells in a culture at a given time-
650 point of incubation retained the ability to divide readily, i.e. to form colonies in R2A/Luria
651 agar plates after 36 h at 37°C. On the other hand, what percentage of the cell population
652 remained viable or metabolically active was tested by checking the cells' ability to
653 accumulate the non-toxic and non-fluorescent molecule fluorescein diacetate and
654 hydrolyze it to fluorescein, which in turn was detected via flow cytometry (71). Post
655 incubation, cells were precipitated from a 100 mL volume of the test culture by
656 centrifugation at 6000 *g* for 20 minutes at 4°C. The supernatant was discarded and the
657 cell pellet resuspended in 2 mL 0.9% NaCl solution. 4 µl of FDA (Sigma, USA) solution
658 (5 mg FDA mL^{-1} dimethyl sulfoxide) was added to the resuspended solution following
659 which the cells were incubated for 15-20 minutes at 37°C. Incubated cells were washed
660 and resuspended again in 0.9% NaCl solution (final resuspension was done in 500 µl)
661 and analyzed using a FACSVerse flow cytometer (Becton Dickinson, USA).
662 Fluorescence was measured through the excitation wavelengths 475-495 nm and the
663 emission wavelengths 520-530 nm. 10000 randomly-taken cells were analyzed for each
664 sample and dot plots were generated using the fluorescence level of each cell as a
665 function of its forward scattering of a 488 nm wave detected by a photodiode array
666 detector. The data were analyzed using the software BD FACSuite (Becton Dickinson)
667 by specific quadrant gating for each experiment that in turn were predetermined based
668 on unstained samples. In all the individual gating experiments, <2% cells within the
669 FDA-less controls exhibited fluorescence: this was the maximum proportion at which
670 false positives could be there within the different values reported for viable cell
671 percentage.

672 **Determining glycine-betaine concentration in spent medium**

673 After incubation in MST broth for 12 h at 50°C, SMMA_5 or SMMA_5_TC cells were
674 harvested from 100 mL spent medium by centrifugation at 6000 *g* for 20 minutes. The
675 supernatant was lyophilized down to 1 mL and then filtered by passing through a 0.22
676 µm cellulose acetate filter (Sartorius Stedim Biotech, Germany). 25 µL of the filtrate was
677 analyzed by high performance liquid chromatography (HPLC) followed by standard UV
678 detection (72), using a Waters platform that encompassed a FlexInject sample-injector,
679 1525 Binary HPLC pump, a 2998 photodiode array detector, and the software Breeze
680 2.0 (Waters Corporation, USA). Isocratic elution was carried out in a Hypersil SCX
681 column having 5 µm particle size, 250 mm length and 4.6 mm inner diameter
682 (Phenomenex, USA), using a mixture of disodium phosphate buffer (0.05 M, pH 4.7) and
683 methanol (95:5%, v/v) as the mobile phase (injection volume: 25 µL; flow rate: 1 mL
684 minutes $^{-1}$ at 25°C; detection wavelength: 195 nm). 0, 2, 4, 8 and 10 mM glycine-betaine
685 standards (Sigma, USA) were used to generate the calibration curve. Glycine-betaine in
686 the sample was identified by comparing its retention time with that of the standard; purity
687 of chromatographic peaks was checked using Breeze 2.0. Quantification was based on
688 the area of the chromatographic peak using the calibration curve $Y = mX + C$, where Y
689 is the peak-area; X is the concentration of glycine-betaine; m is the slope of the
690 calibration curve; C is the curve intercept for the sample.

691

692 **Analysis of genomes and putative proteomes**

693 The whole genome shotgun sequences of SMMA_5 and SMMA_5_TC were determined
694 using an Ion S5 system (Thermo Fisher Scientific, USA) as described previously (73,
695 74). The two raw read datasets were deposited to the Sequence Read Archive of the

696 National Center for Biotechnology Information (NCBI), USA, under the common
697 BioProject PRJNA296849, with the run accession numbers SRR10281900 and
698 SRR10281899 respectively. The assembled genome sequences were annotated for
699 potential ORFs, or CDSs, using the Prokaryotic Genome Annotation Pipeline of NCBI,
700 and deposited to GenBank with the accession numbers WIAB00000000 and
701 WIAC00000000 respectively. Completeness of the new genomes, or that of the
702 comparator *Paracoccus* species, was determined using CheckM 1.0.12 on the basis of
703 what percentage of the genus-specific marker genes was present in the genome
704 considered (75). Pairwise DNA-DNA hybridization experiments were carried out using
705 the online software Genome-to-Genome Distance Calculator 3.0 (76). Orthologous
706 genes-based average nucleotide identity between genome pairs was calculated using
707 ChunLab's online ANI Calculator (77).

708 To identify potential modifications in the genome of SMMA_5_TC with respect to
709 the SMMA_5 genome, the two sequences were first aligned using the software MAFFT
710 (78) with default parameters. The software SNP-sites (79) was then used to identify
711 single nucleotide polymorphisms (SNPs) from the alignment; in order to enumerate the
712 different transition and transversion events, the VCF file generated was processed with
713 the help of VCFtools (80), while the software SnpEff (81) was used to annotate all the
714 SNPs at the nucleotide as well as translated amino acid sequence levels.

715 The core, and species-specific unique, genes of *Paracoccus* members including
716 SMMA_5 were delineated with the help of the bacterial pan genome analysis pipeline
717 BPGA 1.3 (82) as described previously (14). This analysis involved the SMMA_5
718 genome plus the 44 non-redundant genome sequences of *Paracoccus* species (each
719 having a completeness level of ≥97%) available in the GenBank (Table S1). In the

720 working dataset, one genome sequence was included per species, preferably that of the
721 type strain, unless the same was unavailable in the GenBank. SMMA_5_TC was left out
722 because two sibling genomes with identical gene-contents would have distorted the
723 results pertaining to the identification of unique genes.

724 Putative protein sequence catalogs were derived from the genomes of SMMA_5,
725 SMMA_5_TC and the 44 comparator species of *Paracoccus* which have near-complete
726 genome sequences available in the GenBank. Subsequent to this, the pI profile of each
727 catalog was determined using the software Isoelectric Point Calculator that utilizes the
728 Henderson-Hasselbach equation and calculates pKa values for all the individual protein
729 sequences present in a catalog (83).

730 For SMMA_5 and 14 other *Paracoccus* species occupying the same clade in the
731 phylogenomic tree (Fig. 1), complete gene catalogs were downloaded from GenBank.
732 Within each catalog, genes attributable to COGs of proteins (84) were selected, and
733 then COG-counts under individual functional categories were determined. Whether
734 COG-count under a functional category was significantly high or low in a particular
735 genome was determined by Chi Square test, as described previously (14), using the
736 contingency chart shown in Table S8 (a *P* value less than 0.001 was considered as the
737 cut-off for inferring whether the presence of COGs under a category was high or low for
738 a species). Hierarchical clustering was carried out to quantitatively decipher the
739 relatedness between the genomes in terms of their enrichment of various COG
740 categories (85). The heat map illustrating the results of hierarchical cluster analysis was
741 constructed with the help of an R script using complete linkage method (14).
742
743

SUPPLEMENTAL MATERIAL

Supplemental materials in the form of a Word file named "Supplementary_Information.doc", and an Excel file named "Supplementary_Dataset.xlsx", accompany this paper.

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AUTHOR STATEMENT

WG conceived the research program, designed the experiments, interpreted the results and wrote the paper. NM planned and performed the experiments, harnessed all the modules of the study, and analyzed and curated the data. CR led the studies of genomics and also carried out experiments of physiology. SC, SD and SB carried out the physiological studies while JS performed studies of genomics. RC made critical intellectual contributions to the overall work as well as the paper. All the authors read and vetted the paper. The authors have no conflict of interest to declare.

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1062 **FIGURES**

Figure 1. Phylogenetic relationships delineated based on 92 conserved marker gene sequences for SMMA_5, SMMA_5_TC, and 44 other *Paracoccus* species which have near-complete genome sequences available in the GenBank (see Table S1).

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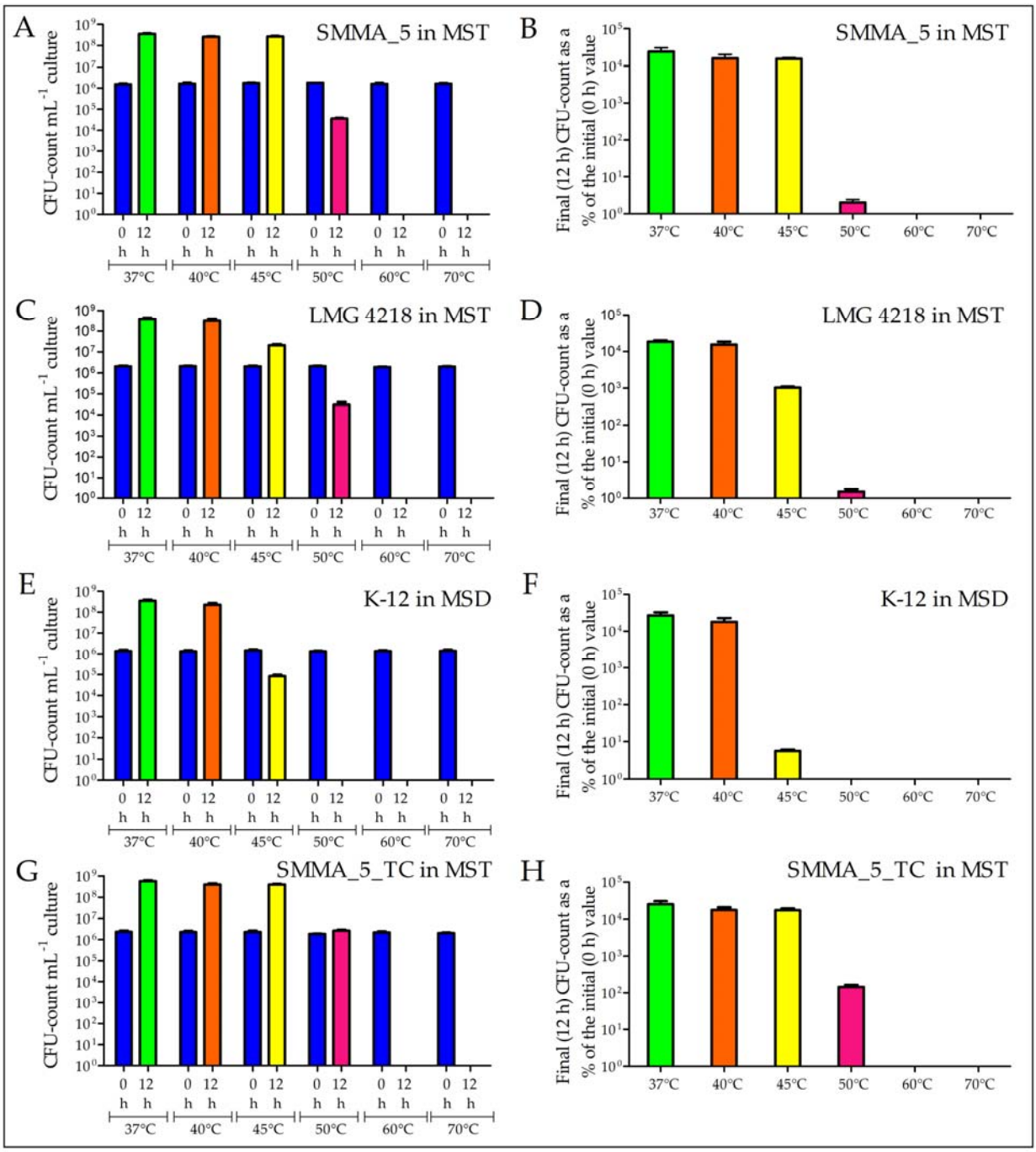

Figure 2. Increase or decrease in the CFU-counts of *Paracoccus* strains SMMA_5, LMG 4218 and SMMA_5_TC in MST, and *E. coli* strain K-12 in MSD, after 12 h of incubation at 37°C, 40°C, 45°C, 50°C, 60°C and 70°C. (**A**) 0 h and 12 h CFU-counts for SMMA_5 recorded at the different incubation temperatures; (**B**) representing each final (12 h)

CFU-count datum of panel A as a percentage of the corresponding initial (0 h) CFU-count; (**C**) 0 h and 12 h CFU-counts for LMG 4218 recorded at the different incubation temperatures; (**D**) each 12 h CFU-count datum of panel C represented as a percentage of the corresponding 0 h CFU-count; (**E**) 0 h and 12 h CFU-counts for K-12 recorded at the different incubation temperatures; (**F**) each 12 h CFU-count datum of panel E represented as a percentage of the corresponding 0 h CFU-count; (**G**) 0 h and 12 h CFU-counts for SMMA_5_TC recorded at the different incubation temperatures; (**H**) each 12 h CFU-count datum of panel G represented as a percentage of the corresponding 0 h CFU-count. All the data shown in this figure are averages obtained from three different experiments; error bars indicate the standard deviations of the data. Across the panels, and irrespective of the bacterium considered, all 0 h data are represented by blue bars, while 12 h data for incubations at 37°C, 40°C, 45°C, 50°C, 60°C and 70°C are represented by green, orange, yellow, pink, red and purple bars respectively.

1065

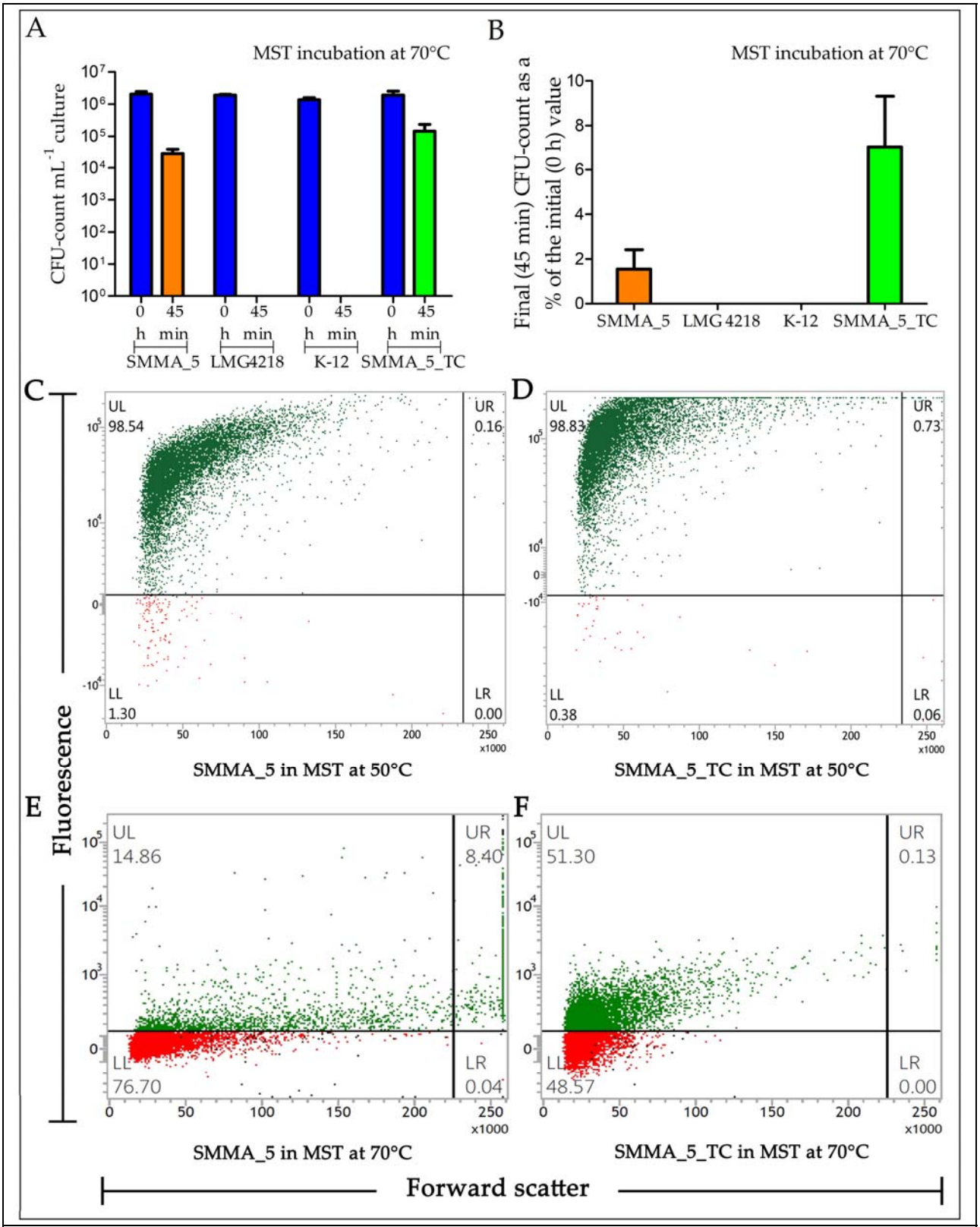

Figure 3. CFU-retention (panels A and B) by *Paracoccus* strains SMMA_5, LMG 4218

and SMMA_5_TC in MST, and *E. coli* strain K-12 in MSD, after 45 minutes of incubation at 70°C. Percentages of cells remaining viable (panels C to F) in MST cultures of SMMA_5 and SMMA_5_TC, after 12 h at 50°C and 45 minutes at 70°C, have also been shown. (**A**) number of CFUs present mL^{-1} of the different broth cultures, at 0 h (blue bars for all the cultures tested) and 45 minutes of incubation at 70°C, for SMMA_5 (orange bar), LMG 4218 (purple bar), K-12 (yellow bar) and SMMA_5_TC (green bar); (**B**) percentages of the initial CFU-counts that were retained in the SMMA_5, LMG 4218, SMMA_5_TC and K-12 after 45 minutes of incubation at 70°C (strain wise color code for the bars are same as that used in A). The data shown in panels A and B are averages obtained from three different experiments; error bars indicate the standard deviations of the data. Flow-cytometry-based dot plots showing the proportions of FDA-stained and FDA-unstained cells for (**C**) SMMA_5 after 12 h at 70C, (**D**) SMMA_5_TC after 12 h at 50°C, (**E**) SMMA_5 after 45 minutes at 70°C, and (**F**) SMMA_5_TC after45 minutes at 70°C. In panels C to F, fluorescence versus forward scatter data for 10000 randomly-taken cells have been potted; green and red dots represent cells that were stained and not stained by FDA respectively. Each flow-cytometry-based experiment was repeated for two more occasions and in every instance <2% deviations were observed from the values shown here for the proportions of FDA-stained and FDA-unstained cells.

1066

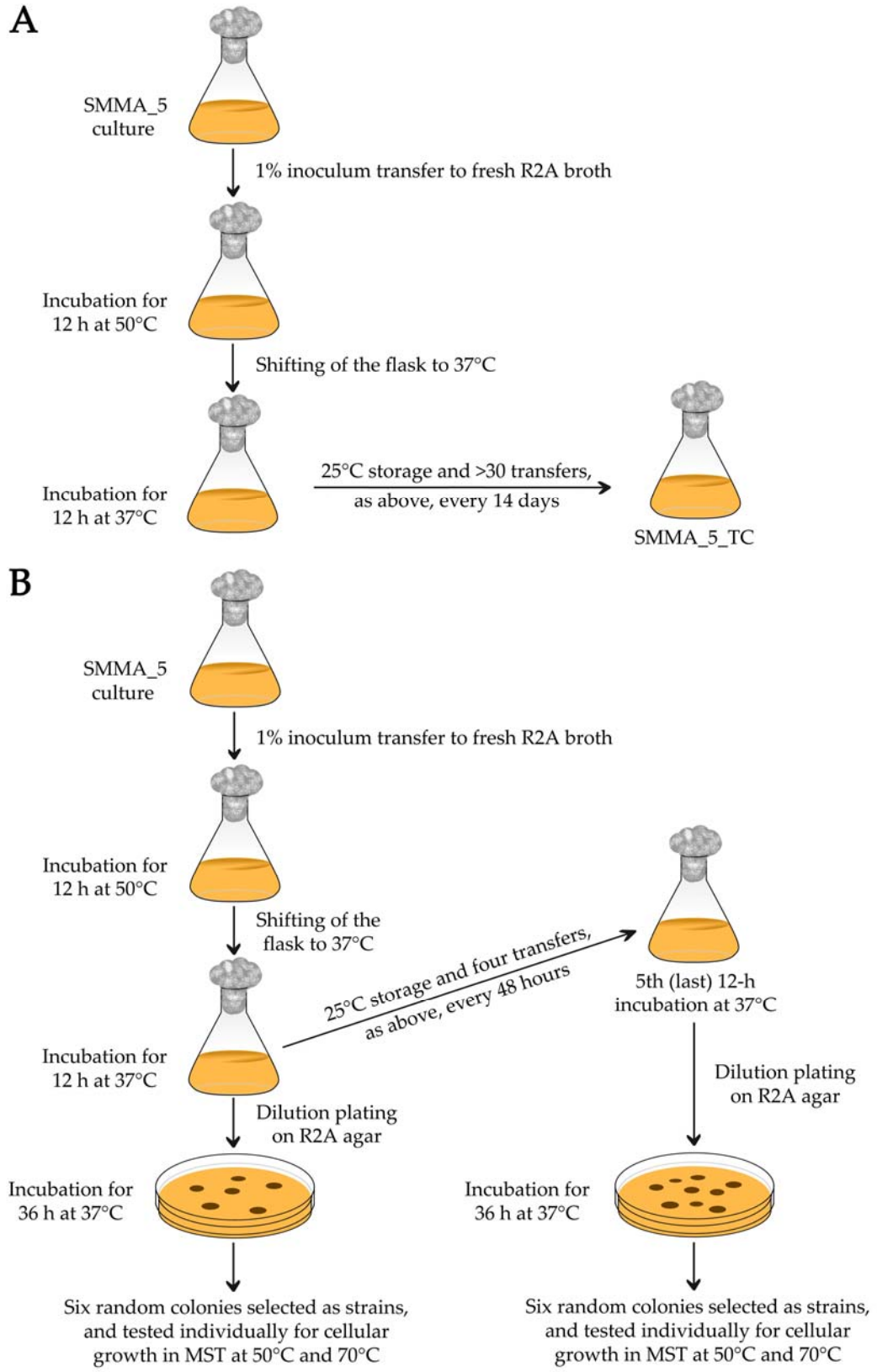

Figure 4. Schematic diagrams showing the procedure followed for thermal conditioning

of SMMA_5. (**A**) steps followed in the creation of SMMA_5_TC; (**B**) steps followed in raising sibling strains of SMMA_5 after one or five cycles (generations) of thermal conditioning.

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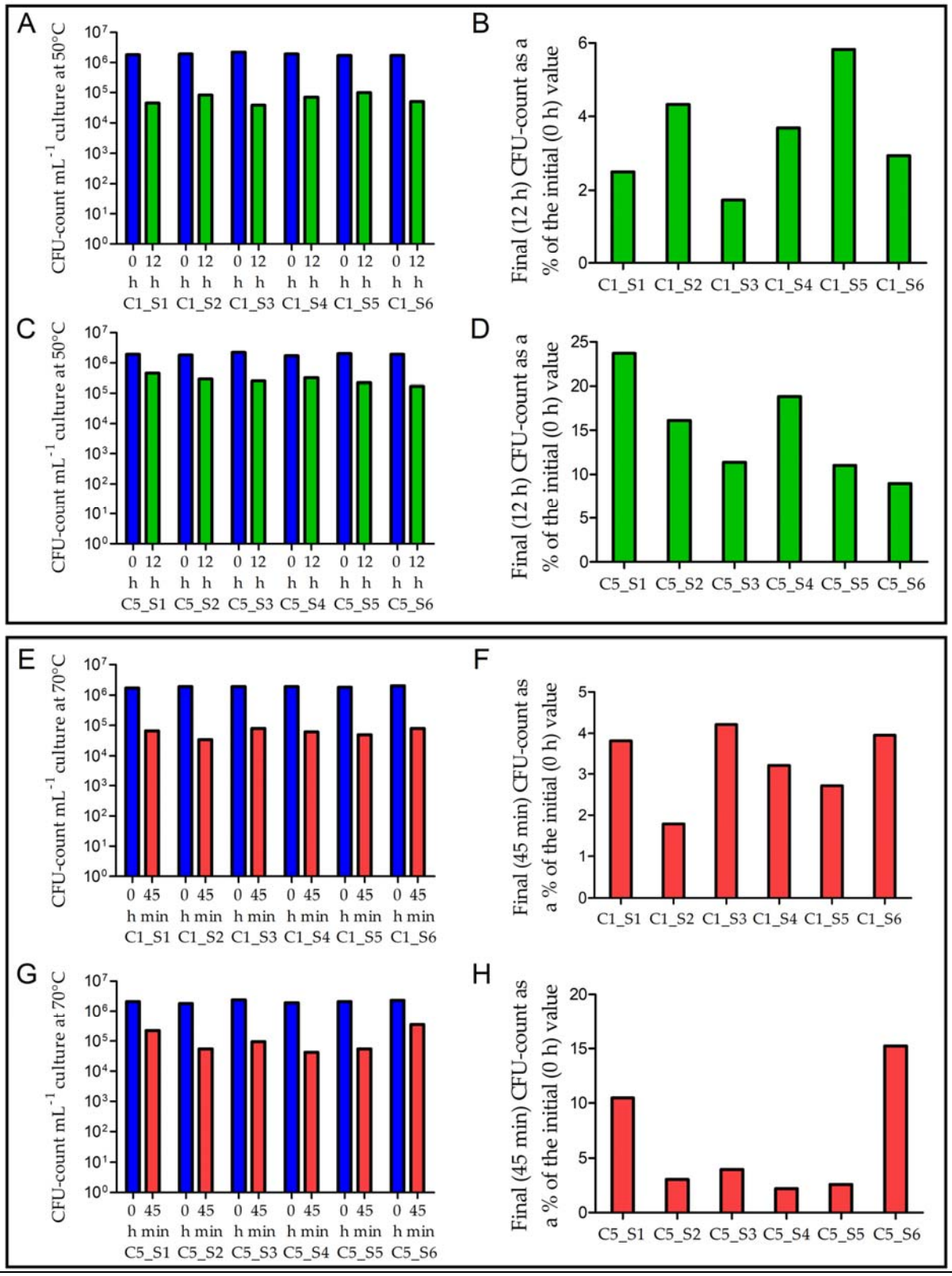

Figure 5. CFU-retention at 50°C (panels A to D) and 70°C (panels E to H) by multiple

sibling strains of SMMA_5 generated after one (panels A, B, E and F) or five (panels C, D, G and H) cycles (generations) of thermal conditioning. (**A**) 0 h and 12 h CFU-counts in MST at 50°C for the six sibling strains of SMMA_5 (C1_S1 to C1_S6) that were raised via a single cycle of thermal conditioning; (**B**) final (12 h) CFU-count of each sibling strain shown in panel A, as a percentage of the corresponding initial (0 h) CFU-count; (**C**) 0 h and 12 h CFU-counts in MST at 50°C for the six sibling strains of SMMA_5 (C5_S1 to C5_S6) that were raised via five cycle of thermal conditioning; (**D**) final (12 h) CFU-count of each sibling strain shown in panel C, as a percentage of the corresponding initial (0 h) CFU-count; (**E**) 0 h and 45 minutes CFU-counts in MST at 70°C for the six sibling strains of SMMA_5 (C1_S1 to C1_S6) that were raised via a single cycle of thermal conditioning; (**F**) final (45 minutes) CFU-count of each sibling strain shown in panel E, as a percentage of the corresponding initial (0 h) CFU-count; (**G**) 0 h and 45 minutes CFU-counts in MST at 70°C for the six sibling strains of SMMA_5 (C5_S1 to C5_S6) that were raised via five cycle of thermal conditioning; (**H**) final (45 minutes) CFU-count of each sibling strain shown in panel G, as a percentage of the corresponding initial (0 h) CFU-count. Across the panels, 0 h, 12 h and 45 minutes data are represented by blue, green and red bars respectively.

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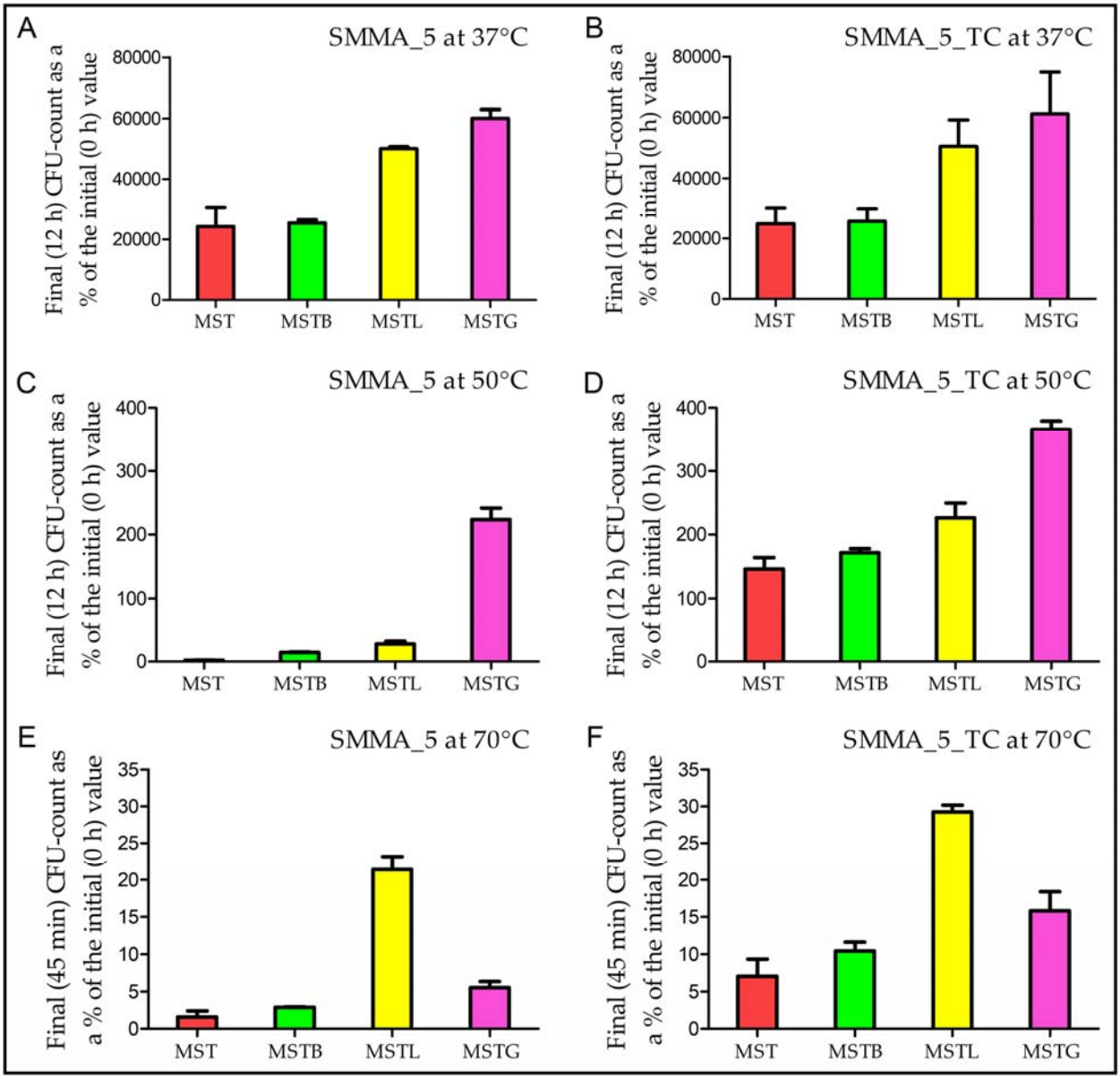

Figure 6. Increase or decrease in the CFU-count of SMMA_5 (panels A, C and E) and SMMA_5_TC (panels B, D and F) in MST, MSTB, MSTL and MSTG media after incubation at 37°C (panels A and B), 50°C (panels C and D) and 70°C (panels E and F). (**A**) final (12 h) CFU-counts of SMMA_5 in the different media at 37°C, represented as percentages of the corresponding initial (0 h) CFU-counts; (**B**) final (12 h) CFU-counts of SMMA_5_TC in the different media at 37°C, represented as percentages of the corresponding initial (0 h) CFU-counts; (**C**) final (12 h) CFU-counts of SMMA_5 in the

different media at 50°C, represented as percentages of the corresponding initial (0 h) CFU-counts; (**D**) final (12 h) CFU-counts of SMMA_5_TC in the different media at 50°C, represented as percentages of the corresponding initial (0 h) CFU-counts; (**E**) final (45 minutes) CFU-counts of SMMA_5 in the different media at 70°C, represented as percentages of the corresponding initial (0 h) CFU-counts; (**F**) final (45 minutes) CFU-counts of SMMA_5_TC in the different media at 70°C, represented as percentages of the corresponding initial (0 h) CFU-counts. The data shown in panels A to F are averages obtained from three different experiments; error bars indicate the standard deviations of the data. Irrespective of the incubation temperature, data recorded for MST, MSTB, MSTL and MSTG media are represented by red, green, yellow and purple bars respectively.

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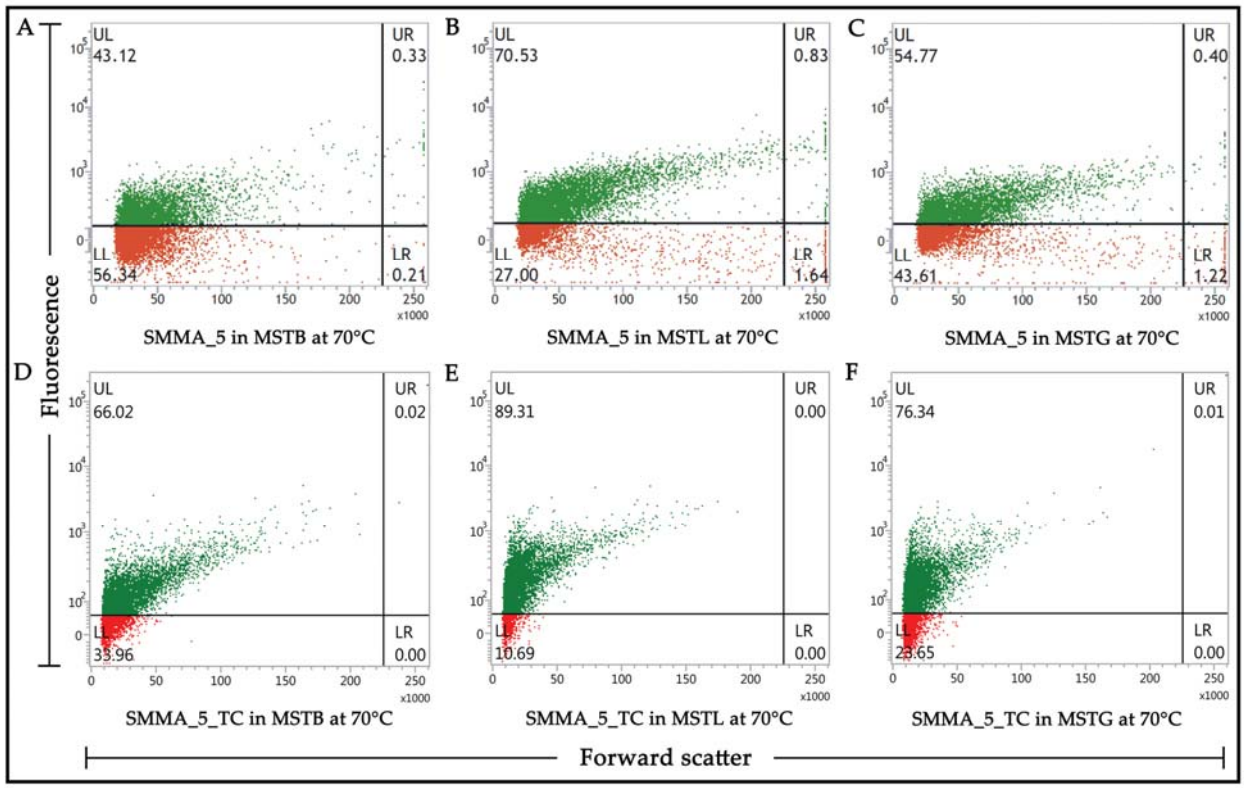

Figure 7. Flow-cytometry-based dot plots showing what percentage of cells remained viable (i.e. the proportion of FDA-stained and FDA-unstained cells) for SMMA_5 (panels A to C) and SMMA_5_TC (panels D to F), after 45 minute incubation at 70°C, in MSTB (panels A and D), MSTL (panels B and E) and MSTG (panels C and F). In each panel, fluorescence versus forward scatter data for 10000 randomly-taken cells have been potted; green and red dots represent cells that were stained and not stained by FDA respectively. Each experiment was repeated for two more occasions and in every instance <2% deviations were observed from the values shown here for the proportions of FDA-stained and FDA-unstained cells.

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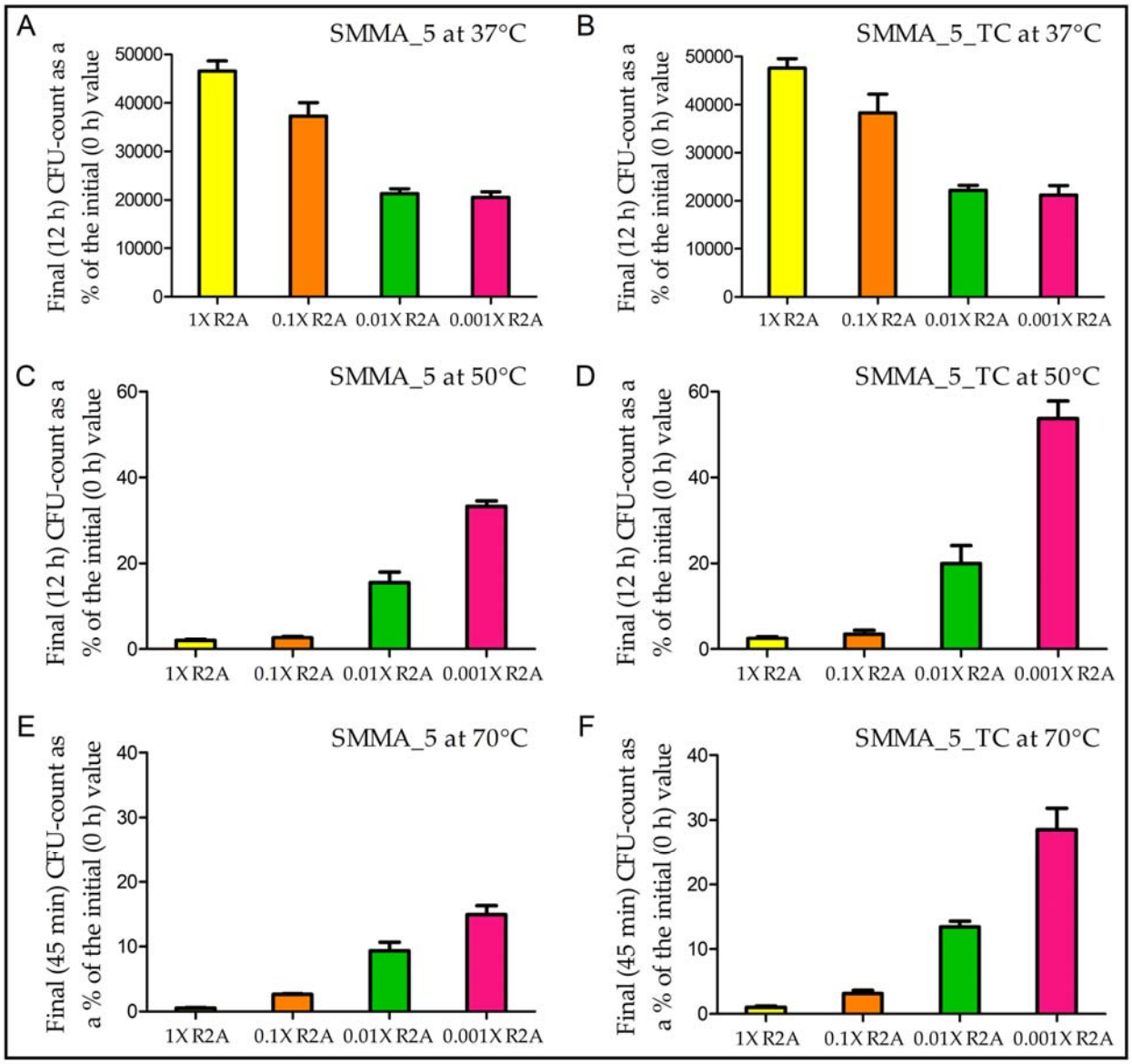

Figure 8. Increase or decrease in the CFU-count of SMMA_5 (panels A, C and E) and SMMA_5_TC (panels B, D and F) in 1X R2A, 0.1X R2A, 0.01 XR2A and 0.001 XR2A media after incubation at 37°C (panels A and B), 50°C (panels C and D) and 70°C (panels E and F). (**A**) final (12 h) CFU-counts of SMMA_5 in the different media at 37°C, represented as percentages of the corresponding initial (0 h) CFU-counts; (**B**) final (12 h) CFU-counts of SMMA_5_TC in the different media at 37°C, represented as percentages of the corresponding initial (0 h) CFU-counts; (**C**) final (12 h) CFU-counts of

SMMA_5 in the different media at 50°C, represented as percentages of the corresponding initial (0 h) CFU-counts; (**D**) final (12 h) CFU-counts of SMMA_5_TC in the different media at 50°C, represented as percentages of the corresponding initial (0 h) CFU-counts; (**E**) final (45 minutes) CFU-counts of SMMA_5 in the different media at 70°C, represented as percentages of the corresponding initial (0 h) CFU-counts; (**F**) final (45 minutes) CFU-counts of SMMA_5_TC in the different media at 70°C, represented as percentages of the corresponding initial (0 h) CFU-counts. The data shown in panels A to F are averages obtained from three different experiments; error bars indicate the standard deviations of the data. Irrespective of the incubation temperature, data recorded for 1X R2A, 0.1X R2A, 0.01 XR2A and 0.001 XR2A media are represented by yellow, orange, green and pink bars respectively.

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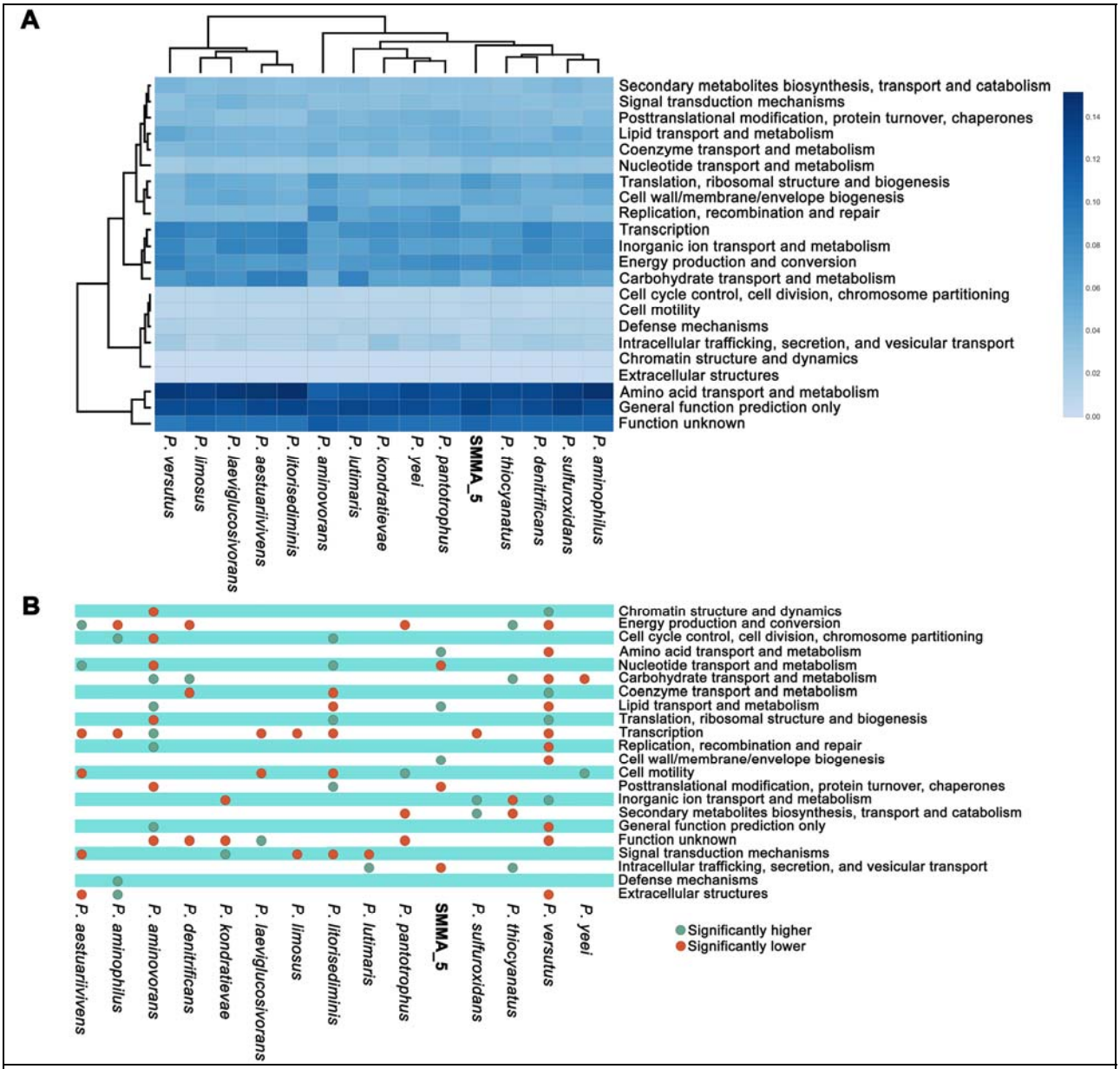

Figure 9. Functional analysis of the genomes of SMMA_5 and its phylogenetically closest *Paracoccus* species (the latter were identified based on the phylogeny of 92 conserved marker gene sequences, Fig. 1). (**A**) Heat map comparing the richness of the metabolic/functional categories across the genomes, determined in terms of the number of Clusters of Orthologous Groups (COGs) of Proteins that are ascribed to the categories in individual genomes; a two-dimensional clustering is also shown, involving

15 bacterial genomes on one hand and the 22 functional categories of COGs on the other; color gradient of the heat map varied from high (deep blue) to low (faint blue), through moderate (light blue), richness of the categories across the genomes. (**B**) Statistically significant, high (green circles) or low (red circles) richness of the functional categories of COG, detected across the genomes via Chi Square test with $p < 0.001$.

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