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1 **Effect of subtherapeutic and therapeutic sulfamethazine concentrations on transcribed genes and**
2 **translated proteins involved in *Microbacterium* sp. C448 resistance and degradation**

3

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16

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19

20 **Abstract**

21 *Microbacterium* sp. C448, isolated from a soil regularly exposed to sulfamethazine (SMZ), can use
22 various sulphonamide antibiotics as the sole carbon source for growth. The basis for the regulation of
23 genes encoding the sulphonamide metabolism pathway, the dihydropteroate synthase sulphonamide
24 target (*folP*), and the sulphonamide resistance (*sul1*) genes, is unknown in this organism. In the present
25 study, the response of the transcriptome and proteome of *Microbacterium* sp. C448 following
26 exposure to subtherapeutic (33 µM) or therapeutic (832 µM) SMZ concentrations was evaluated.

27 Therapeutic concentration induced the highest *sad* expression and Sad production, consistent with the
28 activity of SMZ degradation observed *in cellulo*. Following complete SMZ degradation, Sad production
29 tended to return to the basal level observed prior to SMZ exposure. Transcriptomic and proteomic
30 kinetics were concomitant for the resistance genes and proteins. The abundance of Sul1 protein, 100-
31 fold more abundant than FolP protein, did not change in response to SMZ exposure. Moreover, non-
32 targeted analyses highlighted the increase of a deaminase RidA and a putative sulphate exporter
33 expression and production. These two novel factors involved in the 4-aminophenol metabolite
34 degradation and the export of sulphate residues formed during SMZ degradation, respectively,
35 provided new insights into *Microbacterium* sp. C448 SMZ detoxification process.

36

37 **Keywords:** Sulphonamide antibiotic, *sad* gene, dihydropteroate synthase, omic approaches,
38 deaminase RidA, sulphate exporter

39

40 **Introduction**

41 In commercial food-animal production, antibiotics are used therapeutically, for prophylaxis and, in
42 some jurisdictions, for growth promotion (van Boeckel *et al.* 2017). Following ingestion by farm
43 animals, some antibiotics are excreted intact, and thus animal wastes can represent a major source of
44 antibiotic entry into the environment (Feng *et al.* 2017; Deng *et al.* 2018; Mulla *et al.* 2021).
45 Amendment of crop production systems with manures can lead to the contamination of soil, surface
46 and ground-waters with antibiotics, representing a potential threat to human and environmental
47 health (Spielmeyer *et al.* 2017; Spielmeyer 2018; Ben *et al.* 2019; National Academy of Pharmacy 2019;
48 Duan *et al.* 2022).

49 In France, the sulphonamides are the second most widely used class of antibiotics used in food
50 animal production, representing 19.8% or 73.6 tonnes sold in 2021. Although their use has decreased
51 since the beginning of the 2000s (Anses 2022), their presence in the environment represents a
52 potential risk for ecosystems because of the possible selection and dissemination of sulphonamide-

53 resistance genes and resistant bacteria. Sulphonamides inhibit the dihydropteroate synthase (DHPS,
54 EC 2.5.1.15; encoded by the *folP* gene), a key enzyme involved in the folate biosynthetic pathway in
55 bacteria and some unicellular eukaryotes, by competition with the substrate *p*-amino benzoic acid
56 (Capasso and Supuran 2014). Folate is a precursor for the biosynthesis of tetrahydrofolate, a key
57 coenzyme required for the synthesis of some nucleotides and amino acids. Sulphonamides are
58 bacteriostatic to many Gram-negative and Gram-positive bacteria (Connor 1998). The bacterial
59 resistance is mainly due to mutations in the *folP* gene or the acquisition of alternative genes (*sul1*, *sul2*,
60 and *sul3*) encoding DHPS proteins that do not interact with the sulphonamide antibiotics (Sköld 2000;
61 Perreten and Boerlin 2003; Martin-Laurent *et al.* 2014; Zhou *et al.* 2021).

62 In the environment, the removal of sulphonamides can result from chemical or biological
63 degradation (Chen and Xie 2018; Mulla *et al.* 2021; Hu *et al.* 2022) and is dependent on a variety of
64 factors such as the sulphonamide concentration, and key rate-controlling parameters such as
65 temperature and pH (Cao *et al.* 2019; Chen *et al.* 2019). Different biodegradation pathways have been
66 described for various sulphonamide-degrading bacteria isolated from activated sludge, wastewater or
67 contaminated soil (Chen and Xie 2018; Nunes *et al.* 2020; Perri, Kolvenbach and Corvini 2020). Notably,
68 some *Microbacterium* spp. strains isolated from manured soil, sediment or activated sludge, use
69 sulphonamides as the sole carbon source (Bouju *et al.* 2012; Tappe *et al.* 2013; Topp *et al.* 2013; Kim
70 *et al.* 2019b). Sulphonamide biodegradation by *Microbacterium* spp. requires a gene cluster encoding
71 three proteins; two flavin-dependent monooxygenases, SadA and SadB, possessing an acyl-CoA
72 dehydrogenase domain, and the flavin reductase, SadC (Ricken *et al.* 2017). The latter reduces the
73 flavin mononucleotide (FMN) cofactor required for the activity of the two monooxygenases. The role
74 of the Sad component system in sulphonamide degradation has only been demonstrated *in vitro* using
75 purified enzymes (Ricken *et al.* 2017; Kim *et al.* 2019b). Sulfamethazine (SMZ, also called
76 sulfadimidine), which can reach maximum concentration of 20-25 mg per kg manure or soil (Cycoń *et*
77 *al.* 2019), is transformed by SadA into 4-aminophenol (4AP), the dead-end product 2-amino-4,6-
78 dimethylpyrimidine (ADMP) in stoichiometric amounts and sulphur dioxide (Ricken *et al.* 2017) (Fig.

79 S1). The 4-aminophenol is then transformed by SadB to 1,2,4-trihydroxybenzene which is ultimately
80 mineralized. This degradation pathway is conserved in the Micrococcales order (Kim *et al.* 2019b).

81 In addition to carrying the *sad* gene cluster, *Microbacterium* sp. strain C448 carries the *sul1*
82 gene (Martin-Laurent *et al.* 2014). The relative significance and interplay of the sulphonamide
83 biodegradation pathway and the *sul1* resistance gene in the organism's ability to tolerate and then
84 metabolize the sulphonamides remains unclear.

85 In the present study, transcriptomic and proteomic approaches were used to evaluate the
86 expression levels of the *sad*, *folP* and *sul1* genes, and the abundance of their corresponding proteins.
87 Changes in response to exposure to low subtherapeutic-relevant concentrations and to higher
88 therapeutically-relevant concentrations of SMZ were evaluated. We hypothesized that at low
89 subtherapeutic concentration, *sul1* would not be required to permit biodegradation of the drug
90 whereas at high concentration it would. Time-series transcript- and protein-profiles were analysed and
91 compared to a non-exposed *Microbacterium* culture.

92

93 **Materials and Methods**

94 **Strain and medium**

95 *Microbacterium* sp. strain C448 was isolated from field soil on the Agriculture and Agri-Food Canada
96 research farm in London (Ontario) that was intentionally treated annually for several years with 10 mg
97 SMZ per kg soil. Descriptions of the field, methods for primary isolation, identification and
98 characterization of the isolate are available in Topp *et al.* (2013) and Martin-Laurent *et al.* (2014). The
99 bacterium was cultured in a defined medium (DM) containing glucose (0.5% w/v), amino acids (0.1 g/L
100 of Cys, Trp, Leu, Ile, Val and Met; 0.2 g/L of Arg, and 0.6 g/L of His, Glu), and salts, vitamins and trace
101 elements prepared as described in Amezaga *et al.* (1995). Sulfamethazine sodium salt was purchased
102 from Sigma Aldrich (Saint-Quentin-Fallavier, France; purity \geq 98%). A stock solution (333 mM, 100 g/L)
103 was prepared in water, sterilized by filtration (0.2 μ m) and stored at -20 °C until used.

104

105 **Culture conditions and sample preparation**

106 Transcriptomic and proteomic analyses were performed after cultivation of *Microbacterium* sp. C448
107 strain in DM medium at 28 °C with agitation (150 rpm). The growth was monitored by measuring
108 absorbance at 600 nm (OD₆₀₀). The bacterial cells were collected at the mid-exponential growth phase
109 by centrifugation (5 min, 5,000 x g) and washed twice in DM medium. For each experiment, forty
110 Erlenmeyer flasks (50 and 500 mL) containing respectively 20 mL (transcriptomic) or 180 mL
111 (proteomic) of DM medium, were inoculated with the washed cells at an OD₆₀₀ of 0.4. The flasks were
112 supplemented with 33 μM of SMZ sodium salt (corresponding to 10 mg/L, low concentration, LC), 832
113 μM of SMZ sodium salt (corresponding to 250 mg/L, high concentration, HC) or unsupplemented
114 (Control, no SMZ) before being incubated at 28 °C with agitation (150 rpm) in the dark. A set of four
115 flasks were sacrificed for each concentration immediately after inoculation (T₀), at an early SMZ
116 degradation rate (0.6 h and 1 h post-inoculation (pi)), at an advanced SMZ degradation rate (5 h pi)
117 and after complete SMZ degradation (9 h and 24 h pi) for the transcriptomic and proteomic analyses,
118 respectively. For each sacrificed flask, OD₆₀₀ was measured, and 1 mL of culture was centrifuged (3
119 min, 10,000 x g) to quantify SMZ and ADMP in the supernatant by HPLC. The remaining culture was
120 used for the RNA or protein extractions.

121

122 **SMZ and ADMP quantification by HPLC**

123 The concentrations of SMZ and its dead-end transformation product ADMP were determined by HPLC
124 on an Agilent 1100 apparatus (Agilent Technologies, Courtaboeuf, France) equipped with a reverse-
125 phase column (C18 Zorbax Eclipse Plus column, 75 mm × 4.6 mm, 3.5 μm) at 22 °C and a diode array
126 detector set at λ = 260 nm (SMZ, retention time = 9.3 min) and 298 nm (ADMP, retention time = 1.1
127 min). The mobile phase was composed of aqueous H₃PO₄ (0.01% v/v, pH = 2.9) (A) and acetonitrile (B)
128 at a flow rate of 1 mL/min. Gradient (linear): 0–5 min: 2% B; 5–8 min: 2–30% B; 8–10 min: 30–90% B;
129 10–11 min: 90–100% B; 11–13 min: 100–2% B. Injection volume: 5 μL. Each sample was analysed twice
130 (technical duplicates). SMZ (Sigma Aldrich, purity > 99%) and ADMP (Alfa Aesar, Thermo Fischer

131 Scientific, Waltham, MA, USA; purity 98%) were used as analytical standards. Concentrated solutions
132 (100 μ M and 1 mM, respectively) were prepared in distilled water and diluted to obtain known
133 concentration solutions in order to have a six-point standard curve for each compound at each
134 concentration range (0-100 μ M and 0-1 mM, respectively).

135

136 **Transcriptomic experiment**

137 *RNA extraction and sequencing*

138 RNeasy® Protect Bacteria Mini Kit (QIAGEN, Germantown, MD, USA) was used for the RNA extraction,
139 according to the manufacturer recommendations. Briefly, 2.5 mL of cell suspension were mixed with
140 5 mL of bacterial RNAprotect™ Reagent (QIAGEN) and incubated for 5 min at room temperature before
141 centrifugation (5,000 g, 10 min). The cells were lysed using lysozyme, proteinase K digestion and
142 mechanical disruption by mixing cells with 0.2 g of acid washed beads (212-300 μ m size, Sigma Aldrich)
143 in a FastPrep-24™ classic apparatus (twice 30 s at 6 m/s, MP Biomedicals, Ilkirch, France). RNA was
144 purified on a column treated with DNase. The quality and the quantity of extracted RNA were
145 estimated using the RNA 6000 Nano LabChip® Kit and the 2100 Bioanalyzer following the manufacturer
146 recommendations (Agilent Technologies). For each sample, more than 500 ng of total RNA were sent
147 to GENEWIZ® (Azenta Life Science, Leipzig, Germany) which performed the rRNA depletion, the cDNA
148 synthesis, and the adapter ligation. The obtained library was sequenced in an Illumina® NovaSeq™
149 6000 apparatus (2 x 150 bp paired-end reads, Illumina, San Diego, CA, USA). More than 18 Mreads
150 were generated per sample with a quality score \geq 36.

151

152 *Bioinformatic analysis and data treatment*

153 The reads were first quality-filtered and trimmed using the wrapper script trim galore (v0.6.4) using
154 cutadapt (v2. 6) to trim the reads (Babraham Bioinformatics) (Martin 2011). The reads were then
155 aligned to the genome of *Microbacterium* sp. C448 using hisat2 (v2.2.1) (Kim *et al.* 2019a), and counted

156 using featureCounts (v2.0.1) (Liao *et al.* 2014). The differences of normalized gene expressions
157 between control and SMZ-treated conditions were highlighted using DESeq2 (Love *et al.* 2014). The
158 RNA counting was standardised between samples using Moose2 (polynoMial nOrmalization Of RNA-
159 SEq data), allowing to normalize FPKM (Fragments per kilo-base per million) or RPKM (reads per
160 kilobase per million) values from multiple samples to correct for non-linear artifacts, introduced by the
161 library construction and/or sequencing process (Annergren and Larsson 2016).

162

163 *Data availability*

164 The sequencing data have been submitted to the Sequence Read Archive (SRA, NIH, NCBI), with the
165 project number PRJNA860753.

166

167 **Proteomic experiment**

168 *Protein extraction*

169 The proteins were extracted according to the cell fractionation method described by Esbelin *et al.*
170 (2018) and Santos and Hébraud (2021) with some modifications. For each flask, 180 mL of bacterial
171 culture was centrifuged (5 min, 4,800 x *g*) and the pellet was washed twice in 30 mL of Phosphate
172 Buffered Saline (pH 7.4). Washed pellet was suspended in 4 mL of Tris-EDTA (20 mM Tris; 5 mM, pH
173 7). Bacterial cells were broken by three passages through a French-press cell disrupter (One Shot Cell
174 Disruptor, Constant Systems Ltd., Daventry, United Kingdom) set at a pressure of 2.6 kbar. After
175 centrifugation (13,000 x *g*, 15 min, 4 °C), the supernatants, mainly containing the soluble proteins
176 (cytosol) but also some membrane pieces and their integrated proteins, were recovered and conserved
177 at -20 °C in 50 mL Protein LoBind® Tubes (Eppendorf, Hamburg, Germany). The pellets, containing the
178 proteins of the cell envelope, were washed twice with 5 mL of Tris 40 mM (pH 8.5), suspended in 200
179 to 600 µL of Tris 25 mM (pH 6.8), depending to the pellet viscosity, and conserved at -20 °C in 1.5 ml
180 Protein LoBind® Tubes (Eppendorf). The quantification of the proteins was performed in triplicate

181 following the protocol of the Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Fischer
182 Scientific, Waltham, MA, USA). The absorbance was read at 596 nm using a microplate reader (Thermo
183 Multiskan™ FC, Thermo Fischer Scientific). Short electrophoresis was performed on 12% SDS-
184 polyacrylamide electrophoresis gels to concentrate 10 µg of proteins per sample in the first millimetres
185 of the resolution gel. The concentrated protein band was manually excised with a sterile scalpel blade
186 and washed, reduced, alkylated and digested by trypsin treatment before the nano-LC-MS/MS analysis
187 as detailed in Esbelin *et al.* (2018) and Santos and Hébraud (2021), with some details. The reduction of
188 disulphide bonds was achieved in 10 mM dithiothreitol prepared in 50 mM ammonium bicarbonate
189 buffer and the incubation was carried out for 30 min at 56 °C. The alkylation of proteins was carried
190 out with 55 mM iodoacetamide prepared in 50 mM ammonium bicarbonate buffer for 30 min in
191 darkness. Finally, bands were dehydrated with 100% acetonitrile for 10 min and the liquid was
192 discarded. The proteins were hydrolysed in 600 ng of trypsin in a 50 mM ammonium bicarbonate buffer
193 for 5 h at 37 °C, ensuring that bands were always in liquid by addition of buffer. Peptides were
194 extracted for 15 min in ultrasound bath with 40 µL of acetonitrile/trifluoroacetic acid (TFA) (99.9/0.1
195 v/v). The supernatants were dry concentrated with a SpeedVac® concentrator (Thermo Savant SPD
196 1010, Thermo Fischer Scientific) for 2 h. The volume was adjusted to 40 µL with a solution of
197 Water/Acetonitrile/TFA (95/5/0.05 v/v/v). After 10 min of ultrasonic bath (VWR® USC, Ultrasonic
198 cleaner USC 600TH, Avantor®, Radnor, PA, USA), the entire supernatant was transferred to a glass HPLC
199 vial prior to LC MS/MS analysis.

200

201 *Quantification of proteins by nano-LC-MS/MS and bioinformatic analyses*

202 Peptide mixtures were randomised before being analysed by nano-LC-MS/MS using the RSLC nano
203 Ultimate™ 3000 (Thermo Fischer Scientific) coupled to the Q Exactive HF-X Hybrid Quadrupole-
204 Orbitrap mass spectrometer (MS) (Thermo Fischer Scientific) with a nano-electrospray ion source.
205 Initially, 1 µL of hydrolysate was pre-concentrated and desalted at a flow rate of 30 µL/min on a C18
206 pre-column 5 cm length x 100 µm (Acclaim™ PepMap™ 100 C18, 5 µm, 100 Å nanoViper, Thermo Fisher

207 Scientific) equilibrated with TFA 0.05% in water. In a second step, the concentration column was
208 switched online with a nanoflow analytical C18 column (Acclaim™ PepMap™ 100 - 75 µm inner
209 diameter × 25 cm length; C18 - 3 µm – 100 Å, Thermo Fisher Scientific) equilibrated with a 95% solvent
210 A (99.9% H₂O, 0.1% formic acid) flow at 300 nL/min. The peptides were then separated according to
211 their hydrophobicity with a 55 min gradient of solvent B (99.9% acetonitrile, 0.1% formic acid) from 5
212 to 32%. For MS analysis, eluted peptides were electrosprayed in positive-ion mode at 1.6 kV through
213 a nano-electrospray ion source heated to 250 °C. The mass spectrometer operated in data dependent
214 mode: the parent ion was selected in the orbitrap cell (FTMS) at a resolution of 120,000 and each MS
215 analysis was followed by 18 MS/MS with analysis of the MS/MS fragments at a resolution of 15,000.
216 For raw data processing, MS/MS ion search was carried out with Mascot v2.5.1
217 (<http://www.matrixscience.com>, Matrix Science) against the UniProt reference database of
218 *Microbacterium* sp. C448 (*i.e.* ref_microb_spc448 20190711-3165 sequences) with the following
219 parameters during the request: precursor mass tolerance of 10 ppm and fragment mass tolerance of
220 0.02 Da, a maximum of two missed cleavage sites of trypsin, carbamidomethylation, oxidation of
221 Methionine and deamidation Asparagine and Glutamine set as variable modifications. Protein
222 identification was validated when at least two peptides from one protein showed statistically
223 significant identity above Mascot scores with a False Discovery Rate of 1%. Ion scores was $-10 \log(P)$,
224 where P was the probability that the observed match was a random event. The Mascot score was
225 respectively 14 with an adjusted p -value of 0.05 for supernatants and 34 with an adjusted p -value of
226 0.05 for pellets. The Progenesis® QI for proteomics v4.2 software (Nonlinear Dynamics©, Waters™,
227 Milford, MA, USA) was used for the label-free protein quantitation analysis, with the same
228 identification parameters as described above. All unique validated peptides of an identified protein
229 were included, and the total cumulative abundance was calculated by summing the abundances of all
230 peptides allocated to the respective protein. With the Progenesis® QI software, the proteomic LC-
231 MS/MS data were statistically analysed using the “between subject design” and p -values were
232 calculated by a repeated measures analysis of variance using the normalized abundances across all

233 runs. Obtained data were expressed in arbitrary unit (AU) corresponding to the areas under the peaks
234 obtained by nano LC-MS/MS normalized by the number of cells.

235

236 ~~The SMZ degradation genes and proteins *sadA/SadA* (ORF 2030/UniProt number W0Z5L8), *sadB/SadB*
237 ~~(ORF 2028/W0Z833) and *sadC/SadC* (ORF 2026/W0Z7H5), and the SMZ target genes and proteins:
238 *folP/FolP* (ORF 1952/W0Z6Y9) and *sul1/Sul1* (ORF 2696/W0Z673) were specifically studied.~~~~

239

240 **Statistical analyses**

241 The statistical analyses were performed using R software (The R Foundation). For the transcriptomic
242 data, the package DESeq2 was used. It is based on the use of negative binomial generalized linear
243 models on un-normalized counts. It allows to determine for each open reading frame (ORF) and at
244 each sampling time whether its level of expression is different between the control and LC or HC
245 condition. Since transcriptional regulation is a short-term response and mRNAs are labile, ORF
246 presenting an expression level different by at least by a factor of two in the presence of SMZ in the
247 medium, and a Benjamini-Hochberg adjusted *p*-value (Benjamini and Hochberg 1995) inferior to 0.001,
248 was considered as having a significant differential gene expression level compared to the SMZ-free
249 control (Love *et al.* 2014).

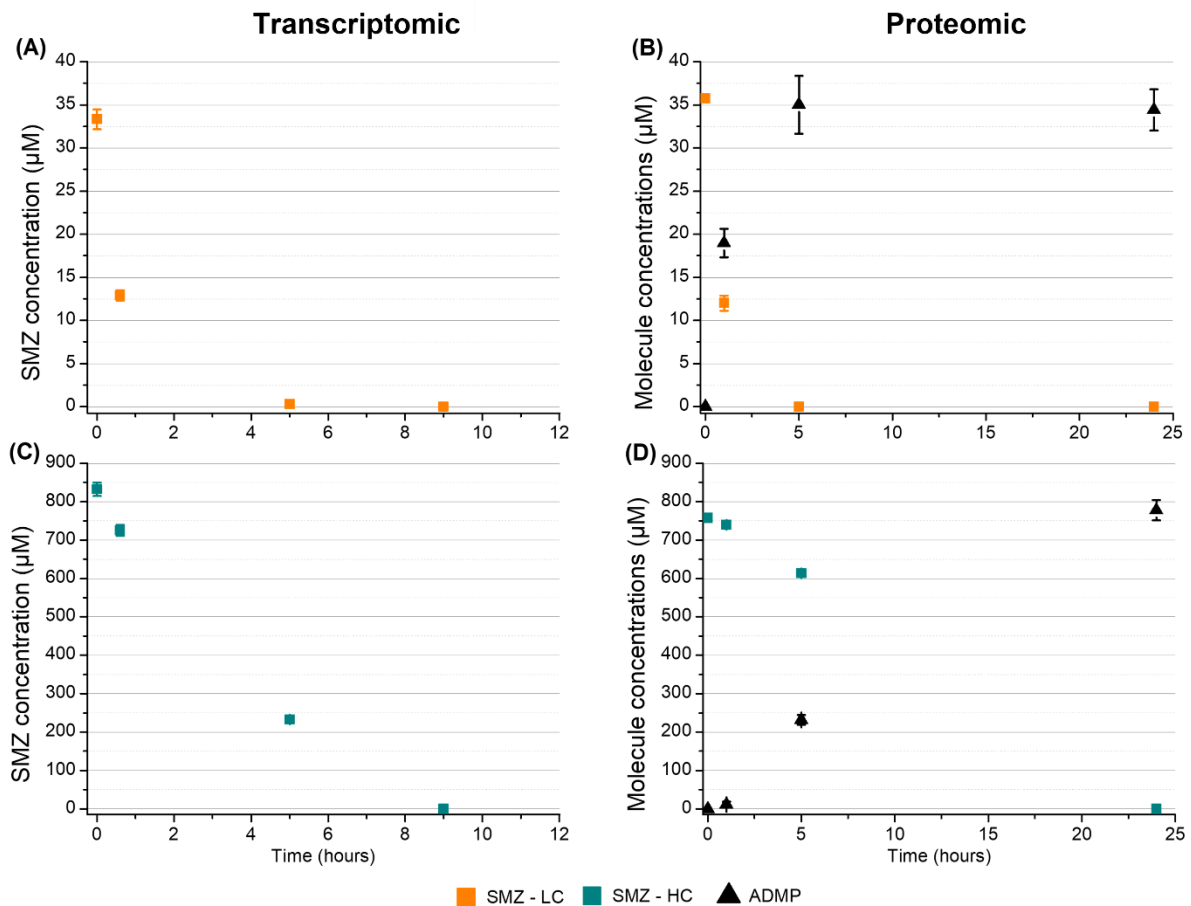
250 For the proteomic data, the assumptions of normality were assessed using several tests (Shapiro-Wilk,
251 Kolmogorov-Smirnov, Jarque-Bera and D'Agostino tests). Box-Cox transformation was applied to
252 achieve normality when appropriate and an ANOVA test followed by Tukey post-hoc were applied with
253 a α risk of 0.05. For the analysis of resistance markers, which turned out to be non-parametric even
254 with the transformation, a Kruskal-Wallis test followed by Dunn's test were applied. The fold-change
255 was calculated at the three sampling time, comparing LC and HC SMZ concentrations *versus* control.
256 For proteomic data, the significant comparisons with a minimum fold-change of ± 1.5 were considered
257 as relevant for the *a priori* analyses on Sad and DHPS proteins, and of 2.0 concerning the analyses on
258 resistance markers without *a priori* consideration.

259

260 **Results and Discussion**

261 **Kinetics of SMZ degradation by *Microbacterium* sp. strain C448**

262 The degradation of SMZ by *Microbacterium* sp. C488 started immediately following the addition of 33
263 μM SMZ, and followed a typical single first-order kinetics (Fig. 1A and 1B). The degradation rate was
264 very rapid with similar rate constants in the transcriptomic ($k = 0.96 \text{ h}^{-1} r^2 = 0.9753$) and proteomic (k
265 $= 1.1 \text{ h}^{-1} r^2 = 1$) experiments. The SMZ was completely degraded within 5 h of incubation. Under the
266 HC condition (Fig. 1C and 1D), the degradation kinetics were rather different between both
267 experiments. The transcriptomic experiments showed a disappearance of 600 μM (72%) of the initial
268 SMZ concentration at 5 h pi, and complete degradation at 9 h pi (Fig. 1C). For the proteomic
269 experiment, almost 20% of SMZ (146 μM) was dissipated in 5 h pi and complete SMZ degradation
270 observed at 24 h pi (Fig. 1D). As measured in the proteomic experiments, the ADMP end-
271 transformation product was stoichiometrically produced upon the SMZ degradation, reaching 34 ± 2
272 (Fig. 1B) and $778 \pm 23 \mu\text{M}$ (Fig. 1D) at 24 h pi for low and high SMZ concentrations, respectively. As
273 expected, the strong ability of the *Microbacterium* sp. C448 strain to degrade SMZ is in accordance
274 with literature, other *Microbacterium* strains also having the capacity to degrade sulphonamides
275 (Tappe *et al.* 2013; Birkigt *et al.* 2015; Hirth *et al.* 2016; Ricken *et al.* 2017; Martin-Laurent *et al.* 2019).
276



277

278 **Figure 1. The degradation of SMZ by *Microbacterium sp. C448*.** The SMZ degradation (squares) and
 279 the end-product ADMP production (▲) were quantified by HPLC in the *Microbacterium sp. C448*
 280 cultures treated with the low (LC, 33 μM, ■) or the high (HC, 832 μM, ■) concentrations of SMZ, in
 281 the transcriptomic (A, C) and in the proteomic experiments (B, D). The values are mean ± SD (n = 4).
 282

283 Sad responses to the SMZ biodegradation in *Microbacterium sp. C448*

284 In the absence of SMZ (control), *sadABC* transcripts (Fig. 2A, 2C, and 2E) and the three Sad proteins,
 285 found in the cytosolic fraction, were expressed or produced at baseline levels at the beginning of the
 286 experiment (T0) (3.24 ± 0.06 AU of proteins in mean, Fig. 2B, 2D and 2F).

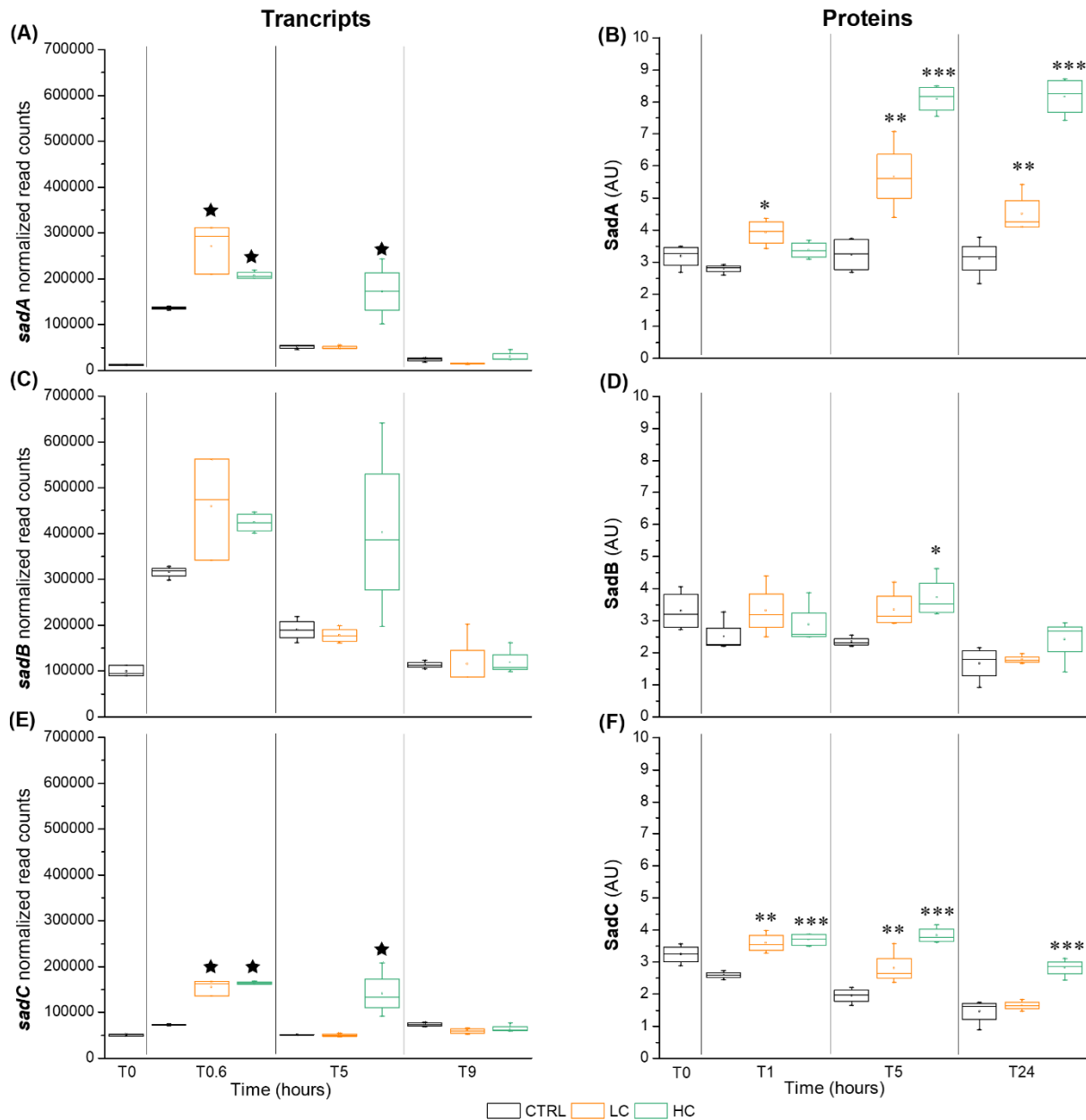
287 In the LC condition, as compared to the control, *sadB* (ORF 2028) and SadB (UniProt number W0Z833)
 288 showed no significant difference in abundance throughout the incubation (Fig. 2C and D). In contrast,
 289 the *sadA* (ORF 2030) and *sadC* (ORF 2026) transcripts increased significantly at 0.6 h pi by a factor of
 290 1.8 and 1.9-fold, respectively (Fig. 2A and 2E, Table S1). Likewise, the abundance of SadA (W0Z5L8)
 291 and SadC (W0Z7H5) protein increased by 1.5-fold at 1 h pi with SMZ ($p = 0.011$ and 0.002 , respectively)

292 (Fig. 2B and 2F, Table S2). From 5 h pi, the abundances of the *sadA* and *sadC* transcripts were the same
293 as the control. However, the abundances of SadA and SadC proteins were still increased at 5 h pi by
294 1.8 and 1.5-fold, respectively ($p = 0.001$ and 0.010). At 24 h pi, the abundance of the SadA protein
295 remained increased by 1.5-fold ($p = 0.009$) while SadC proteins returned to the basal state (Fig. 2B and
296 2F). This slight difference observed between the transcriptomic and proteomic responses might be
297 explained by the fact that the bacterial transcriptome responded more rapidly than the proteome to
298 such an environmental change (Bathke *et al.* 2019).

299 In the HC condition, *sadB* transcript abundance was not changed (Fig. 2C), but the SadB protein
300 abundance increased by 1.6-fold ($p = 0.044$) at 5 h pi (Fig. 2D). The abundances of *sadA* and *sadC*
301 transcripts increased by 1.5 and 2.2-fold, respectively, at 0.6 h pi and by 3.1 and 2.6-fold, respectively,
302 at 5 h pi (Fig. 2A and 2E, Table S1). At 9 h pi, these transcripts did not differ from the control. SadA
303 protein abundance increased by 2.5-fold at 5h pi ($p < 0.001$) and last until the end of experiment (2.6-
304 fold at 24 h pi, $p < 0.001$) (Fig. 2B). SadC protein abundance was increased all along the experiment by
305 in mean 1.8-fold ($p < 0.001$) (Fig. 2F and Table S2). Therefore, while the *sadA* and *sadC* transcript
306 production was transient, decreasing when the concentration of SMZ decreased in the medium, the
307 SadA and SadC proteins persisted.

308 Overall, SadA and SadC proteins were found to be produced *in cellulo* by *Microbacterium* sp. C448
309 concomitantly to SMZ degradation and then continue to accumulate even after complete dissipation
310 of the antibiotic. Moreover, their production depended on the initial exposure concentration to SMZ,
311 suggesting their possible involvement in SMZ biodegradation, as observed before with purified
312 enzymes (Kim *et al.* 2019b).

313



314

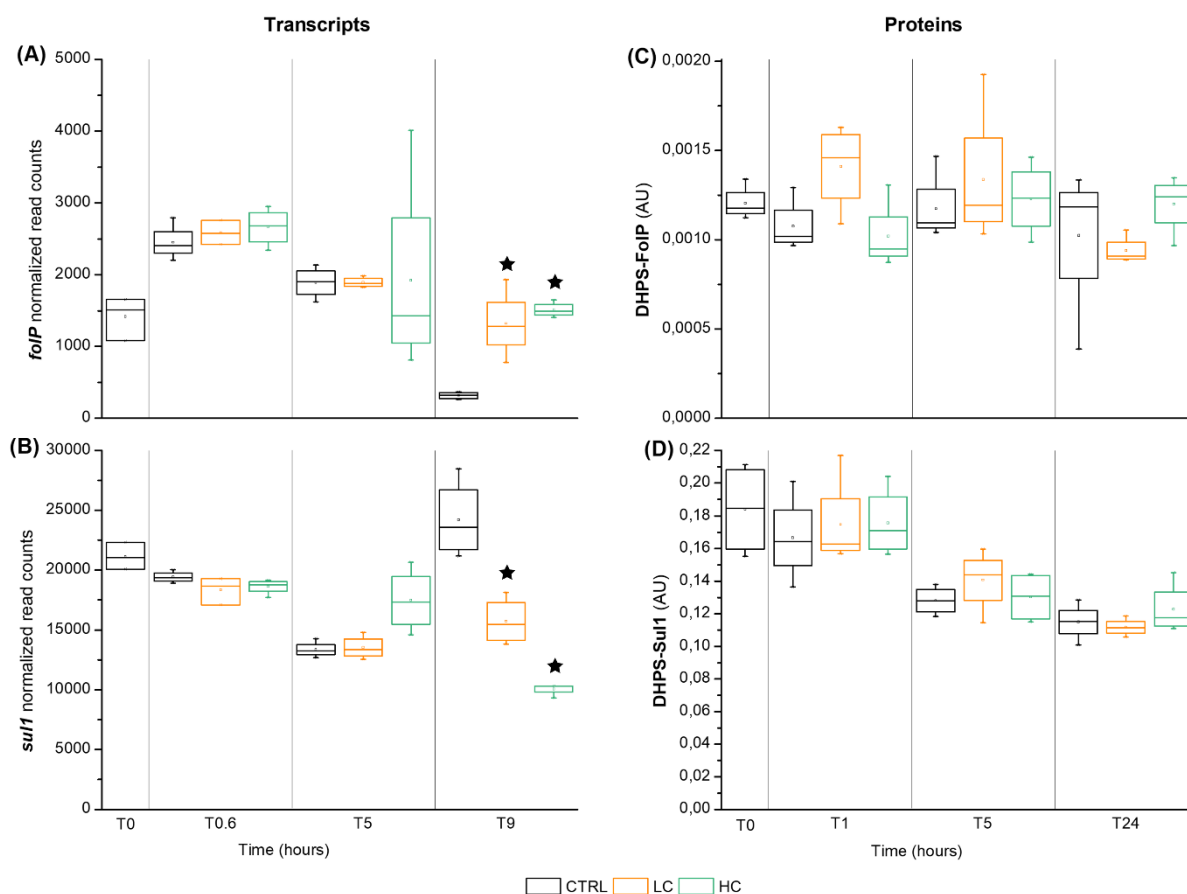
315 **Figure 2. Effect of the SMZ concentration on the abundance of *sad* transcripts and Sad proteins.** The
 316 abundances of *sadA* (A), *sadB* (C) and *sadC* (E) transcripts and of the corresponding proteins (B, D and
 317 F) were reported, in the absence of SMZ (CTRL, □) and after exposure of *Microbacterium* sp. C448 to
 318 a low (LC, □) and a high (HC, □) concentration of SMZ at the different sampling times. Stars and
 319 asterisks represent the significant differences of transcripts (★: $p < 0.001$) and protein abundances (*:
 320 $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$, see Table S1 for details) compared with the control for the
 321 same considered time.

322

323 DHPS gene expression and protein production

324 Numerous studies report the widespread occurrence of *sul1* and *sul2* genes coding for DHPS variants
 325 insensitive to SMZ in antibiotic contaminated environments as substitute of SMZ sensitive DHPS coded

326 by *folP* (e.g. Byrne-Bailey *et al.* 2009; Duan *et al.* 2019; Sardar *et al.* 2021). *Microbacterium* sp. C448
327 harbouring the *folP* and only *sul1* genes (Martin-Laurent *et al.* 2014) like other sulphonamide-
328 degrading Micrococcales (Kim *et al.* 2019b), their regulations were studied in presence of SMZ. The
329 *folP* transcript (ORF 1952) abundance was at least 7-fold lower than that of *sul1* (ORF 2696) for each
330 condition tested here (Fig. 3A and 3B). This trend was even more marked for the abundance of FoIP
331 proteins (W0Z6Y9) detected in the cytosolic fraction which was 100-fold lower than that of Sul1
332 (W0Z673) all along the experiment (Fig. 3C and 3D). During the first five hours of incubation, the
333 abundances of *folP* and *sul1* transcripts were not affected by SMZ neither at LC nor HC. However, at
334 9h pi, the abundance of *folP* transcripts increased by 4.1- and 4.5-fold while the *sul1* transcripts
335 decreased by 2.1 and 3.4-fold in LC and HC conditions, respectively (Table S1). Nevertheless, given the
336 fact that these modifications in the abundances of *folP* and of *sul1* transcripts were observed only at a
337 time point where no more SMZ remained in the medium, one can hypothesise that these modifications
338 were not directly related to SMZ resistance or the metabolite ADMP which was previously shown not
339 to affect the *sul1* gene (Wu *et al.* 2022). The abundance of the FoIP and Sul1 proteins was not affected
340 by SMZ either at LC nor at HC, whatever the sampling time, suggesting that the constitutive expression
341 of *sul1* allows a basal production enough to resist sulphonamide antibiotics (Fig. 3C and 3D, Table S2).
342



343

344 **Figure 3. Effect of the SMZ concentration on the abundance of *folP* and *sul1* transcripts and FolP and**
 345 **Sul1 proteins.** The abundance of *folP* (A) and *sul1* (B) transcripts were determined and the abundances
 346 of their corresponding proteins FolP (C) and Sul1 (D) were quantified in the presence of a low (LC, □)
 347 or a high (HC, □) SMZ concentration and compared with control (CTRL, □) for each sampling time (See
 348 Table S1). Stars represent the significant differences of transcript abundances compared with the
 349 control for the same considered time ($p < 0.001$).

350

351 Search for other genes/proteins potentially involved in the SMZ resistance

352 To further investigate the response of *Microbacterium* sp. C448 to SMZ exposure, other potential
 353 markers of resistance were explored such as those involved in stress response (Dorrian *et al.* 2011; Kim
 354 *et al.* 2017). Those showing a significant response were mainly overexpressed during the SMZ
 355 degradation phase and include chaperones, translation regulation, and DNA replication and repair
 356 factors (Table S3). Only one gene/protein (ORF 2019/W0Z991) was jointly evidenced by transcriptomic
 357 and proteomic data in the cytosolic fraction both in the LC and HC conditions (Table 1), and could have
 358 a preponderant role in the SMZ degradation process. The abundance of the RidA (Reactive

359 intermediate deaminase A) protein family coding gene transcript was increased by 2.3-fold in LC
360 condition at 0.6 h pi and by 2.7- and 3.0-fold at 0.6 and 5 h pi, respectively, in HC condition. It was
361 accompanied by a 2.3-fold increase of the RidA protein abundance at 5 h pi ($p = 0.013$). From 5 h pi,
362 the abundances of the RidA coding gene transcript and its protein were not significantly different from
363 the control. The RidA protein is a putative translation initiation inhibitor of the Rid family (formerly
364 known as YjgF/YER057c/UK114). It leads to an enamine/imine deamination on intermediary
365 metabolites produced during amino acid catabolism and converts them into keto acids, avoiding their
366 accumulation and a metabolic perturbation of the cells (Flynn and Downs 2013; Ernst *et al.* 2014; Irons
367 *et al.* 2020). Moreover, the Rid enzyme family was also described as having the capacity to degrade
368 aromatic compounds, such as 2-aminophenol (Irons *et al.* 2020). The RidA protein family was also
369 suggested to be involved in deamination of 4-aminophenol (4AP) (Rios-Miguel *et al.* 2022), an
370 intermediate in the metabolism of sulphonamides by *Microbacterium* sp. C448. The hypothetical
371 degradation of 4AP by RidA could also explain the low expression/production of SadB in our
372 experiment, due to removal of its substrate (Fig. 2 and S1).

373 Except the stress response, the membrane-based efflux pumps are well known to be another
374 resistance mechanism of bacteria to various antibiotics (Lubelski *et al.* 2007; Handzlik *et al.* 2013;
375 Kumar and Patial 2016; Munita and Arias 2016; Schindler and Kaatz 2016; Greene *et al.* 2018; Reygaert
376 2018). Thus, although the sulphonamides are considered to passively pass through the cellular
377 membrane, and that an uptake through unspecific transporters is not entirely excluded (Zarfl *et al.*
378 2007), the possible involvement of efflux pumps in the resistance of *Microbacterium* sp. C448 to SMZ
379 exposure is unknown. Recently, the increase in the expression/production of ABC transporters was
380 reported in *Escherichia coli* over-expressing *sul1* and *sul2*, suggesting the involvement of transporters
381 in the bacterial resistance to sulphonamides (Zhou *et al.* 2021). In the present study, the exposure of
382 *Microbacterium* sp. C448 to SMZ led to the modulation of 17 transporters in the cell envelope fraction,
383 mainly related to the ABC transporter family. Under LC conditions, there were differences in the
384 abundance of only a few transcripts and proteins (Table 1). At 9 h pi the abundance of one transporter

385 transcript was decreased (ORF 712: X 2.9) and another one was increased (ORF 327: X 2.3), but the
386 abundances of the corresponding two proteins were not modified. However, the abundance of other
387 transporter proteins was increased by 2.3-fold at 1 h pi (W0ZBZ8, $p = 0.018$) or decreased by 6.9-fold
388 at 1 h pi (W0Z6W7, $p = 0.032$) and by 4.1-fold at 5 h pi (W0ZB95, $p = 0.041$). Exposure to antibiotics
389 can cause up- or down-regulation of efflux pumps (Hemmerlin *et al.* 2014).

390 Under HC conditions, much more changes in gene expressions were recorded. At 0.6 h pi, the
391 abundances of five transporter transcripts involved in sulphur, sugar or acetate metabolism were
392 decreased by 2.0 to 2.9-fold while the abundance of one transporter transcript was increased by 2.4-
393 fold (ORF 2808). From 5 h pi, the abundance of these transcripts was not affected by SMZ exposure
394 except that of ORF 3047 which remained decreased by 2.6-fold. In addition, the abundance of three
395 other ABC transporter transcripts probably belonging to glycine betaine transport systems were
396 increased by 2.0 or 2.1-fold only at 5 h pi. Finally, the abundance of ORF 327 transcript was decreased
397 by 2.3-fold at 5 h pi and 2.6-fold overexpressed at 9 h pi. The abundance of one putative ABC
398 transporter protein (W0Z5S8) was decreased by 2.2-fold ($p = 0.013$) at 5 h pi and that of two others
399 W0Z4F3 and W0ZDD8 were increased by 2.2 ($p = 0.024$) and 2.5-fold ($p = 0.032$) at 24 h pi, respectively
400 (Table 1).

401 The combination of transcriptomic and proteomic analysis showed that the high abundance of one
402 transcript (ORF 721) in particular was congruent with that of its related protein (W0Z8D9) in both LC
403 and HC conditions (Table 1). It corresponds to a putative sulphate exporter family. Indeed, the
404 abundance of this transcript was increased in LC conditions by 3-fold at 0.6 h pi and in HC conditions
405 by 3.8-, 13.4- and 74.8-fold at 0.6, 5 and 9 h pi, respectively. Concomitantly, the abundance of the
406 corresponding protein was increased in response to SMZ exposure in HC condition (by 5.4- and 75.8-
407 fold at 5 and 24 h pi, respectively ($p < 0.001$)) (Table 1). This observation suggests that this efflux pump
408 could be involved in the export of the sulphate residues, resulting from SMZ transformation, such as
409 sulphurous acid H_2SO_3 , or sulphuric acid H_2SO_4 (Macris and Markakis 1974; Kim *et al.* 2019b; Yu *et al.*
410 2020). We hypothesize that the up-regulation and -production of a sulphate efflux pump in

411 *Microbacterium* sp. C448 is associated with the removal of sulphur compounds produced by SMZ
412 metabolism that otherwise would reach toxic intracellular concentrations.

413 **Table 1. Genes and proteins potentially involved in *Microbacterium* sp. C448 resistance to SMZ.** The
414 nano-LC-MS/MS identified proteins harbouring a significant difference and with a minimum ± 2.0 -fold
415 change between treated and control conditions are shown. A “/” indicates no differential expression.
416 Not detected indicates the protein abundance was below the detection threshold of the nano-LC-
417 MS/MS; LC and HC: low and high concentrations in SMZ; CTRL: control.

Category	Type	Domain	ORF	Gene name	UniProt code	Characteristics	LC										Proteomic	Transcriptomic	Protec					
							Transcriptomic					Proteomic								Transcriptomic				
							id 4 6	id 4 5	id 4 6	id 4 1	id 4 6	id 4 5	id 4 6	id 4 1	id 4 6	id 4 5				id 4 6	id 4 1	id 4 6	id 4 5	id 4 6
Translation	RidA family protein		2019	-	W02991	Putative translation initiation inhibitor YigF family	+2.3	/	/	/	/	/	/	/	+2.7	+3.0	/	/	+2.3					
Putative sulphate exporter	Uncharacterized		721	-	W028D9	Putative membrane protein YeiH	+3.0	/	/	/	/	/	/	/	+3.8	+13.8	+74.8	/	+5.4					
			712	-	W025S8	Putative protein	/	/	-2.9	/	/	/	/	/	/	/	-2.6	/	-2.2					
		SBP	2176	-	W02B95	NMT1/THI5 like domain protein	/	/	/	/	-4.1	/	/	/	/	/	/	/	/					
			1014	-	W029J1	Nitrate/sulfonate/bicarbonate transporter	/	/	/	/	Not detected	/	/	/	-2.2	/	/	/	Not det					
		Uncharacterized	1016	-	W02A46	Alkanesulfonate transport system permease protein	/	/	/	/	Not detected	/	/	/	-2.3	/	/	/	Not det					
		SBP	2228	<i>opuAC</i>	W02AE6	Proline/glycine betaine transport systems	/	/	/	/	Not detected	/	/	/	+2.1	/	/	/	Not det					
			2230	<i>proV</i>	W02BF3	Glycine betaine transporter	/	/	/	/	/	/	/	/	+2.0	/	/	/	/					
		Uncharacterized	2229	<i>opuAB</i>	W02CI6	Glycine betaine transport system permease protein OpuAB	/	/	/	/	/	/	/	/	+2.0	/	/	/	/					
ABC			585	-	W024F3	Transport permease protein	/	/	/	/	/	/	/	/	/	/	/	/	/					
		TMD	244	<i>ssuC</i>	W02DD8	Alkanesulfonate transporter subunit membrane component	/	/	/	/	/	/	/	/	/	/	/	/	/					
		NBD	265	<i>livF</i>	W02BZ8	Leucine/isoleucine/valine transporter	/	/	/	+2.3	/	/	/	/	/	/	/	/	/					
ATP-binding protein		Uncharacterized	327	<i>drvA</i>	W02CT4	Daunorubicin/doxorubicin resistance	/	/	+2.3	/	/	/	/	/	/	-2.3	+2.6	/	/					
BMP		SBP	533	-	W026W7	Uncharacterized protein	/	/	/	/	-6.9	/	/	/	/	/	/	/	/					
Monosaccharide			2159	-	W02EB5	Cluster maltose/g3p/polyamine/iron - extracellular solute-binding protein	/	/	/	/	/	/	/	/	-2.1	/	/	/	/					
		SBP	2446	-	W024P9	Cluster ribose/xylose/arabinose/galactose	/	/	/	/	Not detected	/	/	/	-2.9	/	/	/	Not det					
APC	SSS	Uncharacterized	3047	<i>actP</i>	W02C79	Cation/acetate symporter	/	/	/	/	/	/	/	/	-2.0	-2.6	/	/	/					
MSF		Uncharacterized	2808	-	W02CN7	Permease of MSF-type transporter	/	/	/	/	Not detected	/	/	/	+2.4	/	/	/	Not det					

419 **Conclusions**

420 In agreement with *in vitro* findings, the congruence of our transcriptomic and proteomic results reveals
421 for the first time the involvement, *in cellulo*, of Sad pathway in the SMZ degradation in *Microbacterium*
422 sp. C448. Contrary to our initial hypothesis whereby *sul1* would be required to permit biodegradation
423 of the drug at therapeutic concentration, we found that the basal expression of *sul1* conferred a
424 natural resistance to *Microbacterium* sp. C448, enough to cope with SMZ. Furthermore, this study has
425 revealed for the first time proteins that may be involved in the metabolism of the key sulphonamide
426 intermediate 4-aminophenol, and the export of inorganic sulphur species that may be toxic if they
427 accumulate in the cell. These conclusions need to be verified and confirmed experimentally. This study
428 contributes to a better understanding of the response of the SMZ-degrading *Microbacterium* sp. C448
429 strain to sulphonamide exposure and provided new insights into SMZ detoxification process.

430

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434

435 **References**

- 436 Amezaga MR, Davidson I, McLaggan D *et al.* The role of peptide metabolism in the growth of *Listeria*
437 *monocytogenes* ATCC 23074 at high osmolarity. *Microbiology* 1995;**141**:41–9.
- 438 Annergren M, Larsson CA. MOOSE2—A toolbox for least-costly application-oriented input design.
439 *SoftwareX* 2016;**5**:96–100.
- 440 Anses. *Surveillance Des Ventes de Médicaments Vétérinaires Contenant Des Antibiotiques En France En*
441 *2021. Rapport Annuel., 2022.*
- 442 Bathke J, Konzer A, Remes B *et al.* Comparative analyses of the variation of the transcriptome and

443 proteome of *Rhodobacter sphaeroides* throughout growth. *BMC Genomics* 2019;**20**:358.

444 Ben Y, Fu C, Hu M *et al.* Human health risk assessment of antibiotic resistance associated with antibiotic
445 residues in the environment: A review. *Environ Res* 2019;**169**:483–93.

446 Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to
447 Multiple Testing. *J R Stat Soc Ser B* 1995;**57**:289–300.

448 Birkigt J, Gilevska T, Ricken B *et al.* Carbon stable isotope fractionation of sulfamethoxazole during
449 biodegradation by *Microbacterium* sp. strain BR1 and upon direct photolysis. *Environ Sci Technol*
450 2015;**49**:6029–36.

451 van Boeckel TP, Glennon EE, Chen D *et al.* Reducing antimicrobial use in food animals. *Science (80-)*
452 2017;**357**:1350–2.

453 Bouju H, Ricken B, Beffa T *et al.* Isolation of bacterial strains capable of sulfamethoxazole
454 mineralization from an acclimated membrane bioreactor. *Appl Environ Microbiol* 2012;**78**:277–
455 9.

456 Byrne-Bailey KG, Gaze WH, Kay P *et al.* Prevalence of sulfonamide resistance genes in bacterial isolates
457 from manured agricultural soils and pig slurry in the United Kingdom. *Antimicrob Agents*
458 *Chemother* 2009;**53**:696–702.

459 Cao L, Zhang J, Zhao R *et al.* Genomic characterization, kinetics, and pathways of sulfamethazine
460 biodegradation by *Paenarthrobacter* sp. A01. *Environ Int* 2019;**131**:104961.

461 Capasso C, Supuran CT. Sulfa and trimethoprim-like drugs – antimetabolites acting as carbonic
462 anhydrase, dihydropteroate synthase and dihydrofolate reductase inhibitors. *J Enzyme Inhib Med*
463 *Chem* 2014;**29**:379–87.

464 Chen J, Jiang X, Tong T *et al.* Sulfadiazine degradation in soils: Dynamics, functional gene, antibiotic
465 resistance genes and microbial community. *Sci Total Environ* 2019;**691**:1072–81.

466 Chen J, Xie S. Overview of sulfonamide biodegradation and the relevant pathways and microorganisms.
467 *Sci Total Environ* 2018;**640–641**:1465–77.

468 Connor EE. Sulfonamide antibiotics. *Prim Care Update Ob Gyns* 1998;**5**:32–5.

469 Cycoń M, Mroziak A, Piotrowska-Seget Z. Antibiotics in the soil environment—degradation and their
470 impact on microbial activity and diversity. *Front Microbiol* 2019;**10**, DOI:
471 10.3389/fmicb.2019.00338.

472 Deng Y, Li B, Zhang T. Bacteria That Make a Meal of Sulfonamide Antibiotics: Blind Spots and Emerging
473 Opportunities. *Environ Sci Technol* 2018;**52**:3854–68.

474 Dorrian JM, Briggs DA, Ridley ML *et al.* Induction of a stress response in *Lactococcus lactis* is associated
475 with a resistance to ribosomally active antibiotics. *FEBS J* 2011;**278**:4015–24.

476 Duan M, Gu J, Wang X *et al.* Factors that affect the occurrence and distribution of antibiotic resistance
477 genes in soils from livestock and poultry farms. *Ecotoxicol Environ Saf* 2019;**180**:114–22.

478 Duan W, Cui H, Jia X *et al.* Occurrence and ecotoxicity of sulfonamides in the aquatic environment: A
479 review. *Sci Total Environ* 2022;**820**:153178.

480 Ernst DC, Lambrecht JA, Schomer RA *et al.* Endogenous synthesis of 2-aminoacrylate contributes to
481 cysteine sensitivity in *Salmonella enterica*. *J Bacteriol* 2014;**196**:3335–42.

482 Esbelin J, Santos T, Ribière C *et al.* Comparison of three methods for cell surface proteome extraction
483 of *Listeria monocytogenes* biofilms. *Omi A J Integr Biol* 2018;**22**:779–87.

484 Feng L, Casas ME, Ottosen LDM *et al.* Removal of antibiotics during the anaerobic digestion of pig
485 manure. *Sci Total Environ* 2017;**603–604**:219–25.

486 Flynn JM, Downs DM. In the absence of RidA, endogenous 2-aminoacrylate inactivates alanine
487 racemases by modifying the pyridoxal 5'-phosphate cofactor. *J Bacteriol* 2013;**195**:3603–9.

488 Greene NP, Kaplan E, Crow A *et al.* Antibiotic resistance mediated by the MacB ABC transporter family:

489 A structural and functional perspective. *Front Microbiol* 2018;**9**:950.

490 Handzlik J, Matys A, Kieć-Kononowicz K. Recent Advances in Multi-Drug Resistance (MDR) Efflux Pump
491 Inhibitors of Gram-Positive Bacteria *S. aureus*. *Antibiot (Basel, Switzerland)* 2013;**2**:28–45.

492 Hemmerlin A, Tritsch D, Hammann P *et al.* Profiling of defense responses in *Escherichia coli* treated
493 with fosmidomycin. *Biochimie* 2014;**99**:54–62.

494 Hirth N, Topp E, Dörfler U *et al.* An effective bioremediation approach for enhanced microbial
495 degradation of the veterinary antibiotic sulfamethazine in an agricultural soil. *Chem Biol Technol*
496 *Agric* 2016;**3**:1–11.

497 Hu J, Li X, Liu F *et al.* Comparison of chemical and biological degradation of sulfonamides: Solving the
498 mystery of sulfonamide transformation. *J Hazard Mater* 2022;**424**:127661.

499 Irons JL, Hodge-Hanson K, Downs DM. RidA Proteins Protect against Metabolic Damage by Reactive
500 Intermediates. *Microb Mol Biol Rev* 2020;**84**:e00024-20.

501 Kim CK, Milheiriço C, De Lencastre H *et al.* Antibiotic resistance as a stress response: Recovery of high-
502 level oxacillin resistance in methicillin-resistant *Staphylococcus aureus* “auxiliary” (fem) mutants
503 by induction of the stringent stress response. *Antimicrob Agents Chemother* 2017;**61**:e00313-17.

504 Kim D, Paggi JM, Park C *et al.* Graph-based genome alignment and genotyping with HISAT2 and HISAT-
505 genotype. *Nat Biotechnol* 2019a;**37**:907–15.

506 Kim DW, Thawng CN, Lee K *et al.* A novel sulfonamide resistance mechanism by two-component flavin-
507 dependent monooxygenase system in sulfonamide-degrading actinobacteria. *Environ Int*
508 2019b;**127**:206–15.

509 Kumar R, Patial SJP. A Review on Efflux Pump Inhibitors of Gram-Positive and Gram-Negative Bacteria
510 from Plant Sources. *Int J Curr Microbiol Appl Sci* 2016;**5**:837–55.

511 Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence

512 reads to genomic features. *Bioinformatics* 2014;**30**:923–30.

513 Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data
514 with DESeq2. *Genome Biol* 2014;**15**:1–21.

515 Lubelski J, Konings WN, Driessen AJM. Distribution and Physiology of ABC-Type Transporters
516 Contributing to Multidrug Resistance in Bacteria. *Microbiol Mol Biol Rev* 2007;**71**:463–76.

517 Macris BJ, Markakis P. Transport and Toxicity of Sulphur Dioxide in *Saccharomyces cerevisiae* var
518 *ellipsoideus*. *J Sci Food Agric* 1974;**25**:21–9.

519 Martin-Laurent F, Marti R, Waglechner N *et al*. Draft genome sequence of the sulfonamide antibiotic-
520 degrading *Microbacterium* sp. strain C448. *Genome Announc* 2014;**2**:e01113-13.

521 Martin-Laurent F, Topp E, Billet L *et al*. Environmental risk assessment of antibiotics in agroecosystems:
522 ecotoxicological effects on aquatic microbial communities and dissemination of antimicrobial
523 resistances and antibiotic biodegradation potential along the soil-water continuum. *Environ Sci*
524 *Pollut Res* 2019;**26**:18930–7.

525 Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads.
526 *EMBnet.journal* 2011;**17**:10–2.

527 Mulla SI, Bagewadi ZK, Faniband B *et al*. Various strategies applied for the removal of emerging
528 micropollutant sulfamethazine: a systematic review. *Environ Sci Pollut Res* 2021, DOI:
529 10.1007/s11356-021-14259-w.

530 Munita JM, Arias CA. Mechanisms of Antibiotic Resistance. *Microbiol Spectr* 2016;**4**, DOI:
531 10.1128/MICROBIOLSPEC.VMBF-0016-2015.

532 National Academy of Pharmacy. *Medicinal Products and Environment.*, 2019.

533 Nunes OC, Manaia CM, Kolvenbach BA *et al*. Living with sulfonamides: a diverse range of mechanisms
534 observed in bacteria. *Appl Microbiol Biotechnol* 2020;**104**:10389–408.

535 Perreten V, Boerlin P. A new sulfonamide resistance gene (*sul3*) in *Escherichia coli* is widespread in the
536 pig population of Switzerland. *Antimicrob Agents Chemother* 2003;**47**:1169–72.

537 Perri R, Kolvenbach BA, Corvini PFX. Subsistence and complexity of antimicrobial resistance on a
538 community-wide level. *Environ Microbiol* 2020;**22**:2463–8.

539 Reygaert WC. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol*
540 2018;**4**:482–501.

541 Ricken B, Kolvenbach BA, Bergesch C *et al.* FMNH2-dependent monooxygenases initiate catabolism of
542 sulfonamides in *Microbacterium* sp. strain BR1 subsisting on sulfonamide antibiotics. *Sci Rep*
543 2017;**7**:1–11.

544 Rios-Miguel AB, Smith GJ, Cremers G *et al.* Microbial paracetamol degradation involves a high diversity
545 of novel amidase enzyme candidates. *Water Res X* 2022;**16**:100152.

546 Santos T, Hébraud M. Extraction and preparation of *Listeria monocytogenes* subproteomes for mass
547 spectrometry analysis. *Methods Mol Biol* 2021;**2220**:137–53.

548 Sardar MF, Zhu C, Geng B *et al.* Enhanced control of sulfonamide resistance genes and host bacteria
549 during thermophilic aerobic composting of cow manure. *Environ Pollut* 2021;**275**:116587.

550 Schindler BD, Kaatz GW. Multidrug efflux pumps of Gram-positive bacteria. *Drug Resist Updat*
551 2016;**27**:1–13.

552 Sköld O. Sulfonamide resistance: mechanisms and trends. *Drug Resist Updat* 2000;**3**:155–60.

553 Spielmeier A. Occurrence and fate of antibiotics in manure during manure treatments: A short review.
554 *Sustain Chem Pharm* 2018;**9**:76–86.

555 Spielmeier A, Höper H, Hamscher G. Long-term monitoring of sulfonamide leaching from manure
556 amended soil into groundwater. *Chemosphere* 2017;**177**:232–8.

557 Tappe W, Herbst M, Hofmann D *et al.* Degradation of sulfadiazine by *Microbacterium lacus* strain

558 SDZm4, isolated from lysimeters previously manured with slurry from sulfadiazine-medicated
559 pigs. *Appl Environ Microbiol* 2013;**79**:2572–7.

560 Topp E, Chapman R, Devers-Lamrani M *et al.* Accelerated biodegradation of veterinary antibiotics in
561 agricultural soil following long-term exposure, and isolation of a sulfamethazine-degrading
562 *Microbacterium* sp. *J Environ Qual* 2013;**42**:173–8.

563 Wu J, Zhang Y, Huang M *et al.* Sulfonamide antibiotics alter gaseous nitrogen emissions in the soil-plant
564 system: A mesocosm experiment and meta-analysis. *Sci Total Environ* 2022;**828**:154230.

565 Yu L, Wang Y, Su X *et al.* Biodiversity, isolation and genome analysis of sulfamethazine-degrading
566 bacteria using high-throughput analysis. *Bioprocess Biosyst Eng* 2020;**43**:1521–31.

567 Zarfl C, Matthies M, Klasmeier J. A mechanistical model for the uptake of sulfonamides by bacteria.
568 *Chemosphere* 2007;**70**:753–60.

569 Zhou Y, Fang J, Davood Z *et al.* Fitness cost and compensation mechanism of sulfonamide resistance
570 genes (*sul1*, *sul2*, and *sul3*) in *Escherichia coli*. *Environ Microbiol* 2021;**23**:7538–49.

571

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573 **Supplemental data**

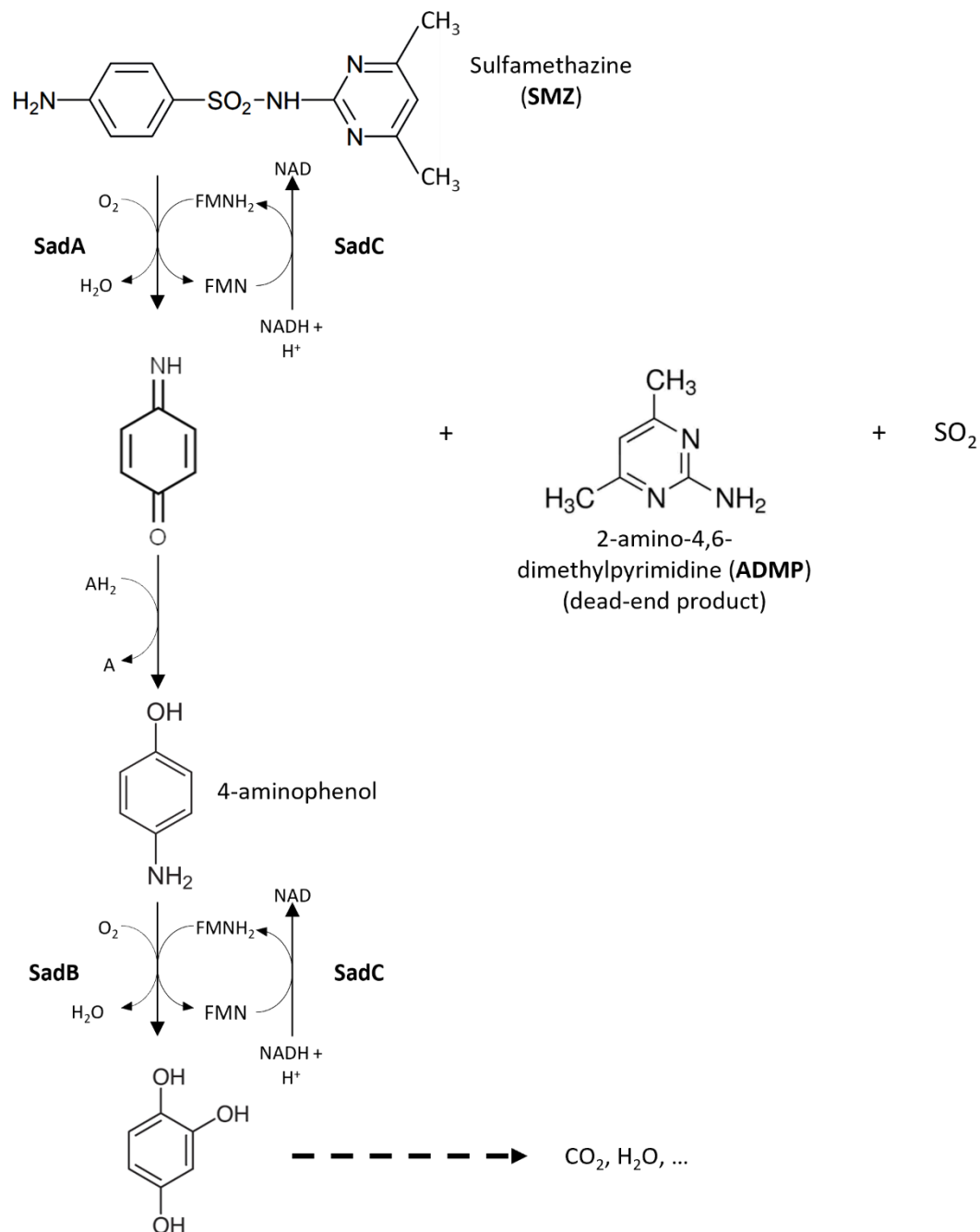
574 **Fig. S1. Proposed SMZ degradation pathway catalysed by Sad proteins in *Microbacterium* sp. C448.**

575 SadA and SadB: monooxygenases; SadC: flavine reductase (according to Ricken *et al.* 2017 and Kim *et*
576 *al.* 2019b).

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582 **Table S1. Modification of the abundance of *sadABC*, *folP* and *sul1* transcripts obtained from DeSeq2**

583 **analysis.** Any statistically significant difference of transcript abundance is expressed as fold-change as

584 compared to control ($n = 4$, $p < 0.001$). CTRL: control; LC: low SMZ concentration; HC: high SMZ

585 concentration; +: increase; -: decrease; NS: not significant.

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Time (h)	Comparison	<i>sadA</i> ORF 2030	<i>sadB</i> ORF 2028	<i>sadC</i> ORF 2026	<i>folP</i> ORF 1952	<i>sul1</i> ORF 2696
0.6	LC vs CTRL	+1.8	NS	+1.9	NS	NS
	HC vs CTRL	+1.5	NS	+2.2	NS	NS
5	LC vs CTRL	NS	NS	NS	NS	NS
	HC vs CTRL	+3.1	NS	+2.6	NS	NS
9	LC vs CTRL	NS	NS	NS	+4.1	-2.1
	HC vs CTRL	NS	NS	NS	+4.5	-3.4

589 **Table S2. Adjusted *p*-values obtained after the Dunn tests was applied on the abundance of Sad and**
590 **DHPS proteins after exposure of *Microbacterium* sp. C448 to SMZ. CTRL: control; LC: low**
591 **concentration; HC: high concentration; +: increase; -: decrease; =: no difference; NS: not significant.**
592 **Bold characters: significant data.**

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Time (h)	Comparison	Sad A W025L8		Sad B W02833		Sad C W027H5		DHPS- <i>folP</i> W026Y9		DHPS- <i>sul1</i> W02673	
		Fold-change	<i>p</i> value	Fold-change	<i>p</i> value	Fold-change	<i>p</i> value	Fold-change	<i>p</i> value	Fold-change	<i>p</i> value
1	LC vs CTRL	+1.5	0.011	+1.3	NS	+1.5	0.002	+1.3	NS	=1.0	NS
	HC vs CTRL	+1.2	NS	+1.2	NS	+1.5	0.000	-1.1	NS	+1.1	NS
5	LC vs CTRL	+1.8	0.001	+1.4	NS	+1.5	0.010	+1.1	NS	+1.1	NS
	HC vs CTRL	+2.5	0.000	+1.6	0.044	+2.0	0.000	=1.0	NS	=1.0	NS
24	LC vs CTRL	+1.5	0.009	+1.1	NS	+1.1	NS	-1.1	NS	=1.0	NS
	HC vs CTRL	+2.6	0.000	+1.4	NS	+1.9	0.000	+1.2	NS	+1.1	NS

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605 **Table S3. List of the stress markers modulated in *Microbacterium* sp. C448 exposed to SMZ.** Any statistically significant modification of transcript abundance
 606 is expressed as fold change as compared to control ($p < 0.001$). The adjusted p -values of proteins are indicated in parentheses.

Category	Type	ORF	Gene name	UniProt code	Characteristics	LC						HC					
						Transcriptomic			Proteomic			Transcriptomic			Proteomic		
						0.6 h pi	5 h pi	9 h pi	1 h pi	5 h pi	24 h pi	0.6 h pi	5 h pi	9 h pi	1 h pi	5 h pi	24 h pi
Translation	RidA family protein	2019	-	W0Z991	Putative translation initiation inhibitor YjgF family	+ 2.3	/	/	/	/	/	+ 2.7	+ 3.0	/	/	+ 2.3 ($p = 0.013$)	/
	Initiation factor IF1	189	<i>infA</i>	W0Z9Z0	-	/	/	- 3.6	/	/	/	/	+ 2.2	- 6.4	/	/	/
	30S ribosomal protein S1	1506	<i>rpsA</i>	W0ZEG5	Cell division-ribosomal proteins cluster	/	/	/	/	/	/	/	+ 2.1	- 2.5	/	/	/
	Ribonuclease P protein component	2283	<i>rnpA</i>	W0ZBK8	Cell division subsystem	/	/	- 3.4	Not detected			/	+ 2.1	- 5.3	Not detected		
Chaperone	Foldase YidC	2285	<i>yidC</i>	W0ZD69	Membrane protein insertase	/	/	/	+ 2.0 ($p = 0.005$)			/	/	/	/	/	/
	Heat shock	2616	-	W0ZER6	18 kDa antigen 2, belongs to the HSP20 family	/	/	/	Not detected			+ 2.2	+ 2.3	/	Not detected		
		251	<i>groS</i>	W0ZAX2	10 kDa family GroES	/	/	/	/	/	/	/	+ 2.5	/	/	/	/
		2931	-	W0Z6T0	60 kDa family GroEL	/	/	/	Not detected			+ 2.4	Not detected				
	Cold shock	2938	-	W0Z7K5	Cold shock domain-containing protein	/	/	/	/	+ 2.0 ($p = 0.032$)			/	/	/	/	/
	Trigger factor	1439	<i>tig</i>	W0Z731	EC:5.2.1.8	/	/	/	/	- 2.6 ($p = 0.024$)			/	/	/	/	/
DNA replication & repair	Primosomal protein	1745	-	W0ZB93	TPR-repeat-containing protein	/	/	/	/	/	/	/	+ 2.5	/	/	/	/
	Single-stranded DNA-binding protein	2306	<i>ssb</i>	W0ZC46	RecFOR pathway	/	/	/	/	/	/	/	+ 2.4	/	/	/	/

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