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

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### ► To cite this version:

Laurianne Paris, Marion Devers-Lamrani, Muriel Joly, Didier Viala, Marie de Antonio, et al.. Effect of subtherapeutic and therapeutic sulfamethazine concentrations on transcribed genes and translated proteins involved in *Microbacterium* sp. C448 resistance and degradation. *FEMS Microbiology Ecology*, 2023, 99 (7), pp.fiad064. 10.1093/femsec/fiad064 . hal-04131399

**HAL Id: hal-04131399**

**<https://hal.inrae.fr/hal-04131399>**

Submitted on 3 Nov 2023

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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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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 (*sulI*) 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  $\mu$ 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 (OD<sub>600</sub>). 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 OD<sub>600</sub> 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 (T<sub>0</sub>), 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, OD<sub>600</sub> 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 H<sub>3</sub>PO<sub>4</sub> (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  $\mu$ 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  $\mu$ 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  $\mu$ 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% H<sub>2</sub>O, 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 *foiP/FoiP* (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).

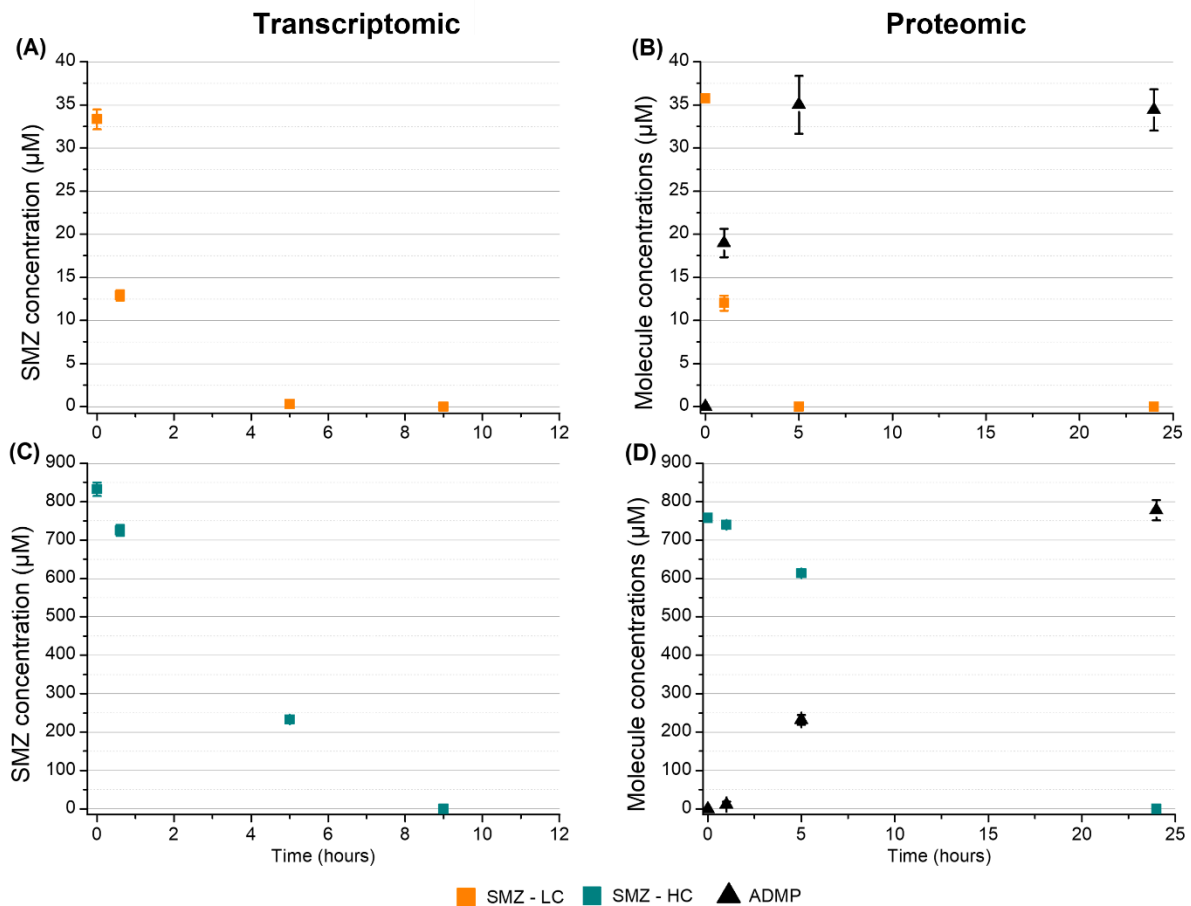
250 For the proteomic data, the assumptions of normality were assessed using several tests (Shapiro-Wilk,  
251 Kolmogorov-Smirnov, Jarque-Bera and D'Agostino tests). Box-Cox transformation was applied to  
252 achieve normality when appropriate and an ANOVA test followed by Tukey post-hoc were applied with  
253 a  $\alpha$  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  $\pm 1.5$  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  $\mu\text{M}$  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  $\mu\text{M}$  (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  $\mu\text{M}$ ) 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  $34 \pm 2$   
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) ( $3.24 \pm 0.06$  AU of proteins in mean, Fig. 2B, 2D and 2F).

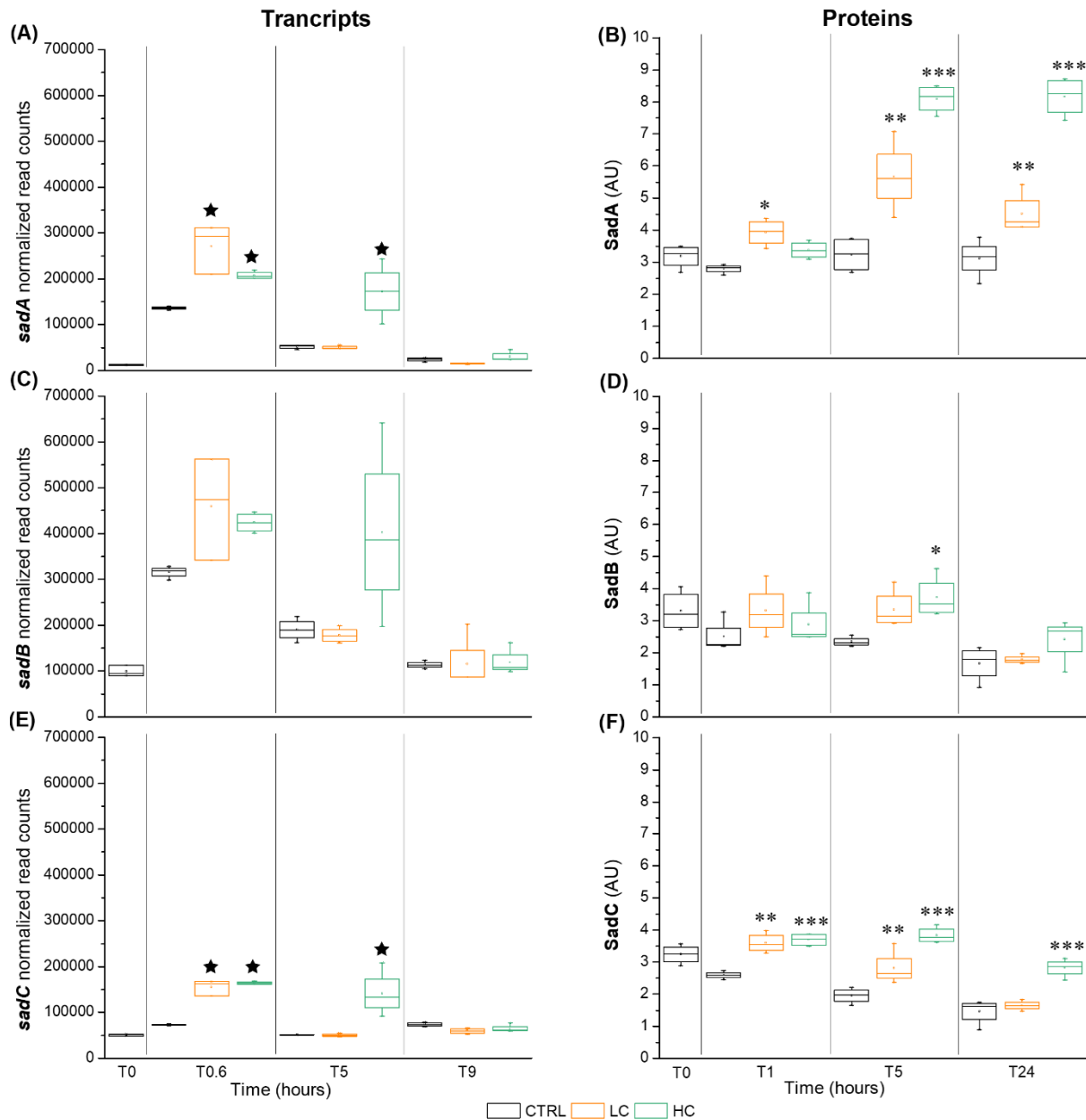
287 In the LC condition, as compared to the control, *sadB* (ORF 2028) and SadB (UniProt number W0Z833)  
 288 showed no significant difference in abundance throughout the incubation (Fig. 2C and D). In contrast,  
 289 the *sadA* (ORF 2030) and *sadC* (ORF 2026) transcripts increased significantly at 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.

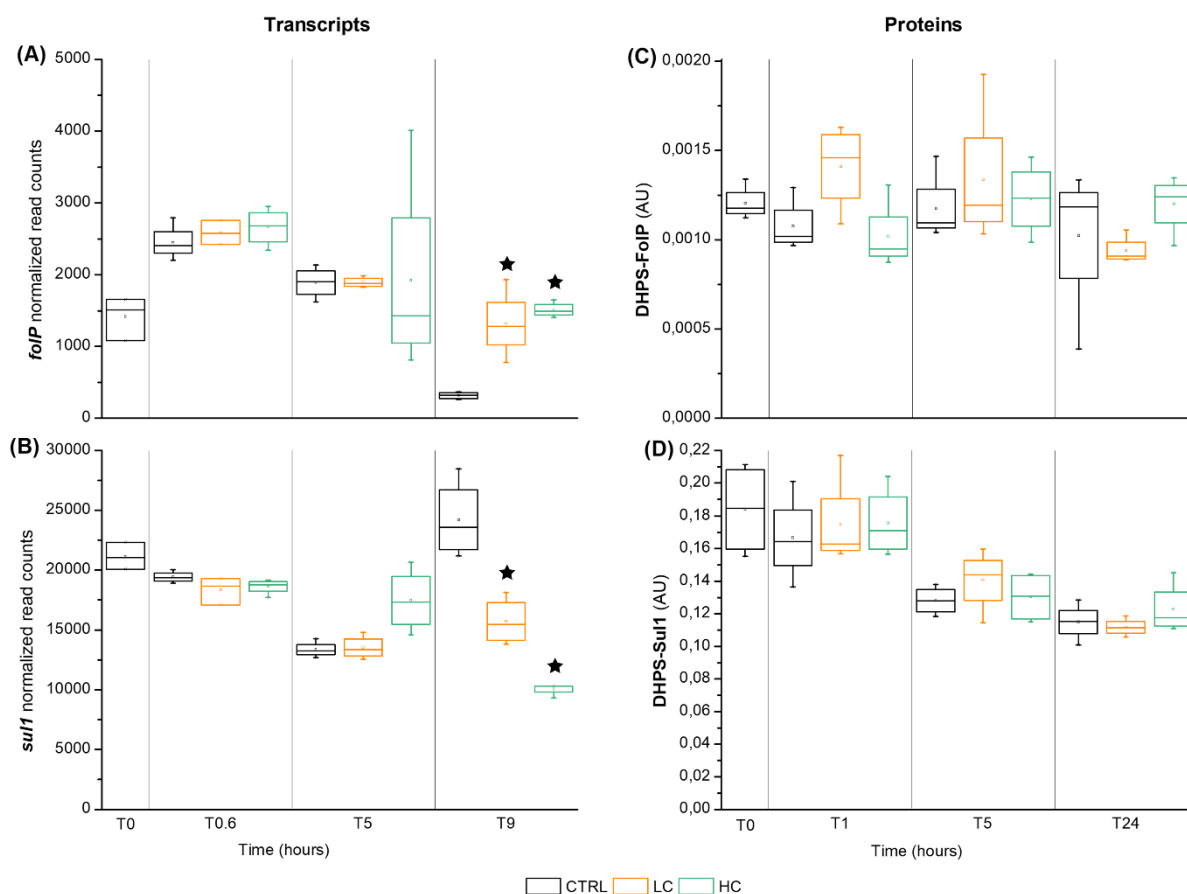
322

### 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  $\pm 2.0$ -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										HC						
							Transcriptomic					Proteomic						Transcriptomic					Protec
							id	5	6	1	6	id	5	6	1	6		id	5	6	0	9	
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

## 431 **Acknowledgments**

432 This work was supported by the Agence Nationale de la Recherche (ANTIBIOTOX project; grant number  
433 ANR-17-CE34-0003).

434

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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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<b>Time (h)</b>	<b>Comparison</b>	<b><i>sadA</i> ORF 2030</b>	<b><i>sadB</i> ORF 2028</b>	<b><i>sadC</i> ORF 2026</b>	<b><i>folP</i> ORF 1952</b>	<b><i>sul1</i> ORF 2696</b>
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	<b>+1.5</b>	<b>0.011</b>	+1.3	NS	<b>+1.5</b>	<b>0.002</b>	+1.3	NS	=1.0	NS
	HC vs CTRL	+1.2	NS	+1.2	NS	<b>+1.5</b>	<b>0.000</b>	-1.1	NS	+1.1	NS
5	LC vs CTRL	<b>+1.8</b>	<b>0.001</b>	+1.4	NS	<b>+1.5</b>	<b>0.010</b>	+1.1	NS	+1.1	NS
	HC vs CTRL	<b>+2.5</b>	<b>0.000</b>	<b>+1.6</b>	<b>0.044</b>	<b>+2.0</b>	<b>0.000</b>	=1.0	NS	=1.0	NS
24	LC vs CTRL	<b>+1.5</b>	<b>0.009</b>	+1.1	NS	+1.1	NS	-1.1	NS	=1.0	NS
	HC vs CTRL	<b>+2.6</b>	<b>0.000</b>	+1.4	NS	<b>+1.9</b>	<b>0.000</b>	+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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