

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22

**Full title: H₂S mediates interbacterial communication through the air reverting
intrinsic antibiotic resistance**

Short title: H₂S producing bacteria can interfere with other species resistance to antibiotics

Thomas-Lopez, Daniel ¹; Carrilero, Laura ¹; Matrat, Stephanie ¹; Montero, Natalia ¹;
Claverol, Stéphane ²; Filipovic, Milos R ³; Gonzalez-Zorn, Bruno ^{1*}

¹ *Departamento de Sanidad Animal (Facultad de Veterinaria) and Centro de Vigilancia
Sanitaria Veterinaria (VISAVET), Universidad Complutense de Madrid, Spain*

² *Plateforme Protéome, Centre Génomique Fonctionnelle de Bordeaux, Université de
Bordeaux, Bordeaux, France*

³ *University of Bordeaux and CNRS; IBGC, UMR 5095, Bordeaux, France*

*Corresponding author: *Universidad Complutense de Madrid, Tel: +34 913943707,
Fax: +34 913943908. Email: bgzorn@ucm.es*

23 Abstract

24 Hydrogen sulfide, a gas classically considered as a by-product of cellular metabolism, is
 25 today recognized as a crucial gasotransmitter in Eukaryotes. Moreover, most bacteria
 26 harbor the eukaryotic orthologous genes for H₂S synthesis, and these genes have been
 27 linked to different metabolic pathways.

28 Some bacteria, however, produce high amounts of H₂S in their extracellular space, a
 29 characteristic classically used for identification purposes. This is the case of *Salmonella*
 30 Typhimurium, which produces H₂S by its *phsABC* operon. Here we show that
 31 extracellular release of H₂S by *S. Typhimurium* is solely dependent on its *phsABC*
 32 operon. Furthermore, we show that *S. Typhimurium* and other H₂S-producing bacteria
 33 can interact with physically distant bacteria through H₂S production. We demonstrate
 34 how H₂S can revert intrinsic cephalosporin resistance of *Enterococcus faecalis* and
 35 *Enterococcus faecium* to complete susceptibility. This study constitutes a significant
 36 step in the study of bacterial interplay and niche competition. Furthermore, as H₂S
 37 releasing drugs have already been designed, our results open the way to future
 38 therapeutic alternatives for the treatment of infections caused by enterococci,
 39 multiresistant pathogens for which no treatments are clinically available.

40

41

42 **Author Summary**

43

44 It has been known for decades that bacteria can communicate with each other through
45 the diffusion of metabolites in the media. However, the capacity of a bacterium to
46 interact with other physically distant cell is a recent discovery of the 21st century. In this
47 work we show how some well-studied bacteria, as it is *Salmonella* spp., interacts with
48 other bacteria thanks to the compound hydrogen sulfide (H₂S) that they produce and
49 release to the environment.

50 In our study we have designed novel techniques that allow us to study the interaction
51 between two bacteria, and we have seen that *Salmonella* is able to affect other species
52 that is even 1 cm away, *i.e.*, a distance corresponding to 10.0000 times its own size.

53 What is more astonishing is that *Enterococcus*, when exposed to the H₂S, is
54 dramatically becomes susceptible to many antibiotics, to which it is supposed to be
55 naturally resistant. *Enterococcus* spp. are responsible for life-threatening infections in
56 hospitals worldwide. Thus, our observations reveal that bacteria can communicate
57 through the air with H₂S, and that this molecule can make bacteria that are highly
58 resistant to antibiotics susceptible to antibiotics, making untreatable infections treatable
59 with current antibiotics.

60

61

62

63

64 **Introduction**

65 It is well established that bacteria can communicate with each other through the
66 diffusion of molecules in the media, what we know as quorum sensing [1]. On the other
67 hand, in the 21st century it has been discovered that physically distant bacteria can also
68 interact with each other or with distant organisms by releasing gaseous molecules [2],
69 many of which were previously considered mere byproducts of bacterial metabolism.
70 Ammonia [3], indole [4], trimethylamine [4] or acetic acid [5] are some these volatile
71 compounds responsible for changes in motility, biofilm formation or antibiotic
72 resistance. In addition, there are a few gases, called gasotransmitters, that are
73 particularly interesting as they also play important roles in eukaryotic cells [6].
74 Gasotransmitters include mainly nitric oxide (NO), hydrogen sulfide (H₂S) and carbon
75 monoxide (CO), and they have been connected to physiological and pathological
76 conditions in cancer [7], the cardiovascular system [8,9], potassium channels [10],
77 cellular ageing [11], animal hibernation [12] and grapes' senescence [13], among others.

78 In microbiology, NO and H₂S have received special attention as they enhance global
79 antimicrobial resistance [14,15]. Bacteria have been shown to possess the orthologous
80 genes to those found in eukaryotic cells for the production of these gases.

81 In the case of hydrogen sulfide, Shatalin *et al.* revealed that this gas confers
82 intracellularly general protection against the bactericidal action of antibiotics through
83 the eukaryotic orthologous pathways cystathionine-β-synthase (CBS), cystathionine-γ-
84 lyase (CSE) or 3-mercaptopyruvate sulfurtransferase (3MST), present in many bacterial
85 families of clinical interest [15].

86 However, H₂S production in large amounts by specific pathways found in certain
87 bacteria, *e. g.* from the *Salmonella*, *Citrobacter*, *Edwardsiella* and *Proteus* genera, has

88 been well known for more than 50 years [16]. Of the different H₂S synthesis
 89 mechanisms in bacteria, the most accurately characterized is the *phsABC* operon of
 90 *Salmonella* Typhimurium, which generates H₂S and sulfite through thiosulfate
 91 reduction. The purpose of this H₂S synthesis by *S. Typhimurium* is not well understood.
 92 It is well established that in the host's gut, thiosulfate can be oxidized to tetrathionate,
 93 which can be used by *S. Typhimurium* for respiratory purposes (reducing it again to
 94 thiosulfate) thanks to the *ttr* genes located in the pathogenicity island 2 (SPI-2) [17].
 95 However, the benefits of a further reduction of thiosulfate to H₂S (instead of using it for
 96 further tetrathionate production) are very scarce [18].

97 Besides, if the function of this gas in bacteria is to enhance antibiotic resistance
 98 [15,19,20], it is intriguing that some species, like the gram positive *Enterococcus*,
 99 inhibit H₂S production by others [21]. However, this suggests that H₂S might be
 100 interacting with neighboring microorganisms, as it is the case with other molecules
 101 already mentioned.

102 Here we characterize the implication of the *phsABC* operon in H₂S synthesis by *S.*
 103 *Typhimurium*. As H₂S produced by the PHS pathway is released extracellularly, we
 104 studied if *S. Typhimurium* could communicate with other bacteria thanks to its H₂S
 105 production. We observed that it is not only the interaction that takes place but that *S.*
 106 *Typhimurium* can also revert other bacteria intrinsic antibiotic resistance, suggesting
 107 niche competition situations can be more complex than expected. Finally, we also
 108 demonstrate that H₂S abolishes antibiotic resistance. As H₂S releasing donors have
 109 already been developed, the combined application of antibiotics with H₂S requires
 110 future in depth studying.

111

112 **Results**

113 **H₂S excretion relies entirely on *phsABC* action**

114 Most bacteria possess H₂S producing genes, namely CBS/CSE and 3MST [15], together
 115 with cysteine desulfhydrases [22]. Nevertheless, a few bacterial species carry, in
 116 addition to the previously mentioned genes, an accessory and specific H₂S producing
 117 pathway. For example, *Salmonella* Typhimurium harbors the *phsABC* operon. To
 118 understand the role of this pathway, we deleted this operon (Fig 1A) and we measured
 119 the H₂S production of the deletion mutant Δphs , both in aerobic and anaerobic
 120 conditions.

121 Kligler media, which in the presence of H₂S forms a dark precipitate, turned completely
 122 black when *S. Typhimurium* wild-type strain was inoculated, whereas Δphs did not
 123 produce any black pigmentation of the media at all (Fig 1B).

124 Lead (II) acetate paper strips, which detect H₂S through the formation of black lead
 125 sulfide, allowed us to detect H₂S released not only to the media, but also outside of the
 126 media, as the strips were located above the culture medium. Again, we observed that the
 127 strips turned completely black with the WT culture, while incubation of Δphs strain
 128 barely caused any staining (Fig 1C).

129 In both cases, complementation of the strain with the pSB74 plasmid, which will be
 130 called pH₂S as it bears the entire *phsABC* operon [23], restored H₂S production at WT
 131 levels in the new strain *S. Typhimurium* Δphs^c (Fig 1).

132

133

134

135

136

137 **The PHS pathway is neither involved in growth nor antibiotic resistance in *S.***

138 ***Typhimurium***

139 H₂S has been linked to antibiotic resistance, via the H₂S synthesis pathways found in

140 most bacteria [15,20]. However, this has not been demonstrated for the *S. Typhimurium*

141 specific PHS pathway, even though this is considered the main source of H₂S in this

142 species [23,24]. Therefore, we carried out various antimicrobial susceptibility tests (S1

143 Fig, **Error! Reference source not found.**S1 and S2 Tables), but no differences were

144 observed in any case. Growth curves showed no difference either in growth rate

145 between WT and Δphs strain (S1 Fig).

146

147

148 **Remote action of *S. Typhimurium* on the intrinsic antibiotic resistance of**

149 ***Enterococcus***

150 H₂S produced by means of the PHS pathway by *S. Typhimurium* is largely released

151 from the cell and does not appear to be implicated its own growth or antibiotic

152 resistance. Thus, we hypothesized that H₂S may have an external role on the antibiotic

153 resistance pattern of neighboring bacteria when *S. Typhimurium* interacted with them.

154 We designed a model to effectively assess signaling between physically distant bacteria

155 (Fig 2). This method consists in preparing antibiograms of the species to be tested and

156 confronting them against another Petri dish in which the H₂S producing strain has been

157 plated with a cotton swab. Subsequently, plates were incubated for a maximum of 24

158 hours.

159

160 *S. Typhimurium* did not significantly alter bacterial growth, hemolysis capacity,
161 colonies' size and morphology or the antibiotic sensitivity profile of the species
162 analyzed, which includes methicillin-resistant *Staphylococcus aureus*, *Bacillus cereus*,
163 *Escherichia coli*, *Proteus vulgaris* and the *S. Typhimurium* Δphs mutant itself (data not
164 shown).

165 However, when faced with *S. Typhimurium*, *E. faecalis*, a major nosocomial pathogen
166 worldwide, became completely susceptible against cephalosporins, drugs to which
167 *Enterococcus* is intrinsically resistant (Fig 3).

168

169 **The *phsABC* operon of *Salmonella* is responsible for the reversion of *Enterococcus***
170 **intrinsic antibiotic resistance**

171 To distinguish if enterococci lose their cephalosporin resistance due to H₂S released by
172 *S. Typhimurium* or by an unrelated mechanism, we confronted *E. faecalis* with the
173 deletion mutant Δphs , which we have shown not to release any H₂S.

174 By performing E-test, we observed that *E. faecalis* Minimal Inhibitory Concentration
175 (MIC) for cefotaxime was dramatically decreased, from higher than 256 mg/L to 3
176 mg/L, when faced specifically with *S. Typhimurium* WT, *i. e.*, the H₂S producing strain.
177 On the other hand, the MIC was not affected when faced with *S. Typhimurium* Δphs
178 strain (Fig 4).

179 Δphs mutant recovered its lethal action against *E. faecalis* when was complemented *in*
180 *trans* (Table 1), which means that it is specifically via the H₂S produced by the *phsABC*
181 operon that *S. Typhimurium* induces *Enterococcus* killing. *S. Typhimurium* (pTrc99a),

182 *i.e.*, the strain carrying the empty vector pTrc99a, was used as a negative control to
183 ensure that this plasmid had no influence on the effect. As we established that *phsABC*
184 is a predominant mechanism for extracellular H₂S release from this species, we
185 conclude that this operon is crucial for the remote induced killing of neighbor bacteria
186 by *S. Typhimurium*.

187

188 **Table 1. Confronting experiments of *E. faecalis* with *S. Typhimurium* WT and its**
189 **respective mutants.**

190

Strains	+ T-S 2 mM			+ T-S 20 mM			+ Cys 2 mM			+ T-S 20 mM Anaerobiosis		
	ZOX	CTX	CEC									
<i>E. faecalis</i>	6	6	16		-		-			-		
vs. <i>S. Typhimurium</i> WT	<u>28</u>	<u>30</u>	19	<u>25</u>	<u>33</u>	16	<u>27</u>	<u>30</u>	17	<u>22</u>	<u>26</u>	19
vs. <i>S. Typhimurium</i> Δphs	6	6	14	6	6	12	<u>27</u>	<u>30</u>	17	6	6	14
vs. Δphs (pTrc99a)	6	6	14	6	6	13	<u>23</u>	<u>23</u>	16		ND	
vs. Δphs (pH ₂ S)	<u>25</u>	<u>28</u>	16	<u>32</u>	<u>34</u>	16	<u>25</u>	<u>27</u>	17		ND	

191 ND: not determined

192 *E. faecalis* JH2-2 is plated on Tryptone-Soya Agar (TSA). *S. Typhimurium* strains are plated on
193 TSA supplemented with thiosulfate 2 or 20 mM. Halos are expressed in mm. Inhibition zones
194 displayed only in the presence of H₂S are underlined. Ceftizoxime (ZOX), Cefotaxime (CTX),
195 Cefaclor (CEC).

196

197

198

199 **The *phsABC* operon is sufficient to induce cephalosporin susceptibility in *E.*** 200 ***faecalis***

201 To identify if the product of the *phsABC* operon was having an effect in adjuvancy with
202 other factors from *S. Typhimurium*, we transformed *E. coli* MG1655 strain with the
203 pH₂S plasmid. We show that the *phsABC* operon is sufficient for the increased
204 production of H₂S by *E. coli* MG1655 (Fig 5), implying that no further networks or
205 genes present in *Salmonella* are necessary. Lead (II) acetate paper strips were stained in
206 a similar way in *E. coli* (pH₂S) and *S. Typhimurium*. When *E. coli* (pH₂S) was
207 incubated in Kligler media, we observed a lesser amount of staining than in *S.*
208 *Typhimurium*. This can be explained by the fact that *S. Typhimurium* also harbors the
209 anaerobic sulfite reductase (*asr*) that generates further H₂S from the reduction of the
210 sulfite previously generated by PHS.

211 Furthermore, when we confronted different *E. coli* strains with *E. faecalis*, we observed
212 that only *E. coli* (pH₂S) could induce the susceptibility of *E. faecalis* to cephalosporins
213 (Fig 3), showing that the *phsABC* function is sufficient for the resistance reversion
214 phenomenon carried out by H₂S and that no secondary metabolites produced by *S.*
215 *Typhimurium* are necessary.

216 We also tested if our observations could be dependent on the presence of oxygen.
217 However, the confrontations experiments performed in aerobiosis and anaerobiosis
218 showed similar results (Table 1), discarding therefore that our results are connected to
219 an augmentation of the reactive oxidative species.

220

221

222 **H₂S is responsible for the reversion of the intrinsic resistance to cephalosporins in** 223 ***E. faecalis***

224 The deletion of the *phsABC* operon of *S. Typhimurium* could have further consequences
225 on the bacteria (*e. g.*, affecting other metabolic pathways) that could ultimately be
226 responsible for the distant effect observed on *Enterococcus* rather than the H₂S itself.
227 To rule out this hypothesis, we tested other sources of H₂S. First, we carried out
228 confrontation experiments in the presence of cysteine, the substrate of the 3MST
229 pathway [15], present in many bacteria including *S. Typhimurium*. Even if we have
230 proved that under basal conditions PHS is the predominant mechanism for extracellular
231 production of H₂S, we hypothesized that addition of external cysteine should increase
232 the production of H₂S by this pathway, as high levels of cysteine are toxic for the cell
233 [22]. Upon adding cysteine, we observed that H₂S is again detected extracellularly in
234 the Δ *phs* mutant (Fig 1) and that this mutant is able to revert *Enterococcus*
235 cephalosporin resistance in a similar extent as the wild-type *S. Typhimurium* (Table 1).
236 We confronted *E. faecalis* with *Proteus vulgaris*, another species commonly known for
237 its extracellular production of H₂S [22] and *P. vulgaris* proved to be equally efficient in
238 inducing cephalosporin susceptibility in *E. faecalis* (Fig 3).

239 Finally, we checked if pure H₂S from a chemical origin also acts synergistically with
240 cephalosporins. NaHS is a widely used H₂S source [15,25]. We designed a technique in
241 which we placed NaHS physically separated from the enterococci. We put our
242 antibiograms in a glass chamber, next to an empty Petri dish in which we placed 0.1
243 grams of NaHS and 2 mL of ultrapure water. Once we sealed the chamber, we gently
244 shook it to put the crystal into contact with the water so that the gaseous H₂S could be
245 released into the chamber (Fig 6).

246 With this experiment, we proved that the resistance of *E. faecalis* to cephalosporins
247 could be reversed by pure H₂S. In addition, we also showed that other *E. faecalis* strains
248 are affected in the same way. Most notably, V583, the first vancomycin resistant clinical
249 isolate, displayed the same phenotype (Fig 7).

250

251 ***E. faecium* cephalosporin resistance is also reverted in the presence of H₂S**

252 Next, we were interested in testing the effects of H₂S on the other pathogenic
253 enterococcus, *E. faecium*, as in the last decade this species has acquired even higher
254 clinical relevance than *E. faecalis*, partially because most of the enterococcal
255 vancomycin resistant clones currently detected are *E. faecium* [26]. We have verified
256 that this species is also susceptible to the synergic effect of H₂S with cephalosporins
257 (Fig 8), further suggesting the potential application of this combination in clinical
258 settings.

259

260 **Reversion of the intrinsic resistance of enterococci in the presence of H₂S takes** 261 **place specifically with methoxy-imino cephalosporins**

262 As we can notice in Figs 3, 7 and 8, and Table 1, synergy between H₂S and
263 cephalosporins does not take place with every compound of this family of antibiotics.
264 By performing antibiograms of a set of 20 cephalosporins, including 1st to 4th generation
265 cephalosporins, we have observed that in the presence of H₂S *E. faecalis* demonstrates a
266 significant susceptibility against cefotaxime, ceftriaxone, cefuroxime, cefpodoxime and
267 ceftizoxime (Table 2). These drugs, frequently used in clinical settings, contain a
268 common methoxy-imino group in their structure (Fig 9), which is not present in other

269 cephalosporins within the family, suggesting that this motif might be a key in the
270 synergy displayed between H₂S and cephalosporins.

271

272 **Table 2. Antibigrams of *E. faecalis* V583 strain, in the presence and absence of NaHS 0.1**
273 **grams.**

	TSA	H ₂ S 0.1 g
274		
	Cefaclor 30	10
275	Cefadroxil 30	13
	Cephalexin 30	8
276	Cefalotin 30	16
	Cefamandole 30	14
	Cefazolin 30	14
277	Cefepime 30	13
	<u>Cefotaxime 30</u>	6
278	Cefotetan 30	6
	Cefoxitin 30	6
	Cefpirome 30	22
279	<u>Cefpodoxime 10</u>	6
	Cefprozil 30	21
	Cefradine 30	12
280	Cefsulodin 30	6
	<u>Ceftizoxime 30</u>	6
281	<u>Ceftriaxone 30</u>	6
	<u>Cefuroxime 30</u>	6

282 Methoxy-imino cephalosporins are underlined.

283

284 **H₂S and cephalosporins kill enterococci**

285 Finally, to further characterize the H₂S-cephalosporins synergy, *E. faecalis* JH2-2 was
286 grown in liquid media in the presence and absence of H₂S and/or cefotaxime (CTX).

287 By performing a Two-way ANOVA ($\alpha = 0.05$; $P < 0.001$; $F = 68,46$; $df = 17, 28$) and a
288 post-hoc Tukey–Kramer analysis, used for single-step multiple comparison of all pair of
289 means, we observed that there is a strong synergistic effect between H₂S and CTX ($P <$

0.001), not only by inhibiting bacterial growth in the presence of both compounds, but also by producing a bactericidal effect (Fig 10, S4 Table). With this analysis we confirmed that, in the concentration range used in our study, H₂S does not affect bacterial growth on its own. Even if enterococci were resistant to cephalosporins, a minor effect of cefotaxime on bacterial growth was noticeable, but this is something that could be observed with many antibiotic discs in antibiograms or other antibiotic susceptibility tests.

Preliminary experiments with cefaclor, a non-methoxy-imino cephalosporin, or with nalidixic acid, a quinolone against which *E. faecalis* is resistant, showed no synergy with H₂S (data not shown), indicating that the displayed phenotype is specific of certain antibiotics.

301

302 **The persulfidome analysis of *E. faecalis* reveals potential targets for H₂S**

We carried out random transposition mutagenesis of *Enterococcus* under cephalosporin selective pressure, constructing a high-density transposon mutant library. However, no mutants were obtained using this approach. This suggests that a single gene alteration is not enough to prevent resistance reversion, or that this alteration has to take place in an essential gene. Therefore we wanted to address posttranslational changes of the protein that are induced by H₂S.

Signaling by H₂S is now widely linked to an oxidative posttranslational modification of cysteine residues (RSH) called persulfidation (RSSH) (alternatively S-sulphydration) [27–29]. Exposure of cells to H₂S results in an increase of protein persulfidation [27,30]. We performed biotin-tagging assay (BTA) to extract total persulfidated

313 proteins [31,32]. As shown in Fig 11, an increase of protein persulfidation could be
314 observed in bacteria treated with H₂S.

315 The proteins were subjected to electrophoretic separation, followed by trypsin digestion
316 and LC/MS/MS analysis. 66 cysteine-containing proteins were identified as
317 persulfidated (Table 3, S7 Table). Among them, peptide ABC transporters, as well as
318 proteins of unknown function are potential targets of H₂S. Despite not being
319 quantitative, to best of our knowledge, this is the first report of a persulfidome in
320 bacteria. Further studies will be needed to determine the protein, or combination of
321 proteins, that are responsible for the susceptibilization of enterococci to cephalosporins
322 when exposed to H₂S.

323

324

325

326

327

328

329

330

331

332

333 **Table 3. Persulfidome of *E. faecalis*.** List of proteins of *E. faecalis* JH2-2 detected to interact
334 with H₂S.

335

Accession	Gene	Description
Q833I9	EF1963	Phosphoglycerate kinase
Q839G8	EF0201	Elongation factor Tu
Q834N1	EF1613	Formate acetyltransferase
Q836Q8	EF1050	DNA-binding response regulator
Q833I8	EF1964	Glyceraldehyde-3-phosphate dehydrogenase
H7C7A0	EF2739	Alkyl hydroperoxide reductase, C subunit
Q837D6	EF0907	Peptide ABC transporter, peptide-binding protein
Q82ZF4	EF3106	Peptide ABC transporter, peptide-binding protein
P23530	EF0710	Phosphoenolpyruvate-protein phosphotransferase
Q839G9	EF0200	Elongation factor G
Q833J0	EF1962	Triosephosphate isomerase
Q831U9	EF2398	30S ribosomal protein S2
Q836C4	EF1191	DegV family protein
Q839C1	EF0255	L-lactate dehydrogenase 1
Q836R2	EF1046	Pyruvate kinase
Q836Z4	EF0949	Phosphotransacetylase
Q837P9	EF0784	S-adenosylmethionine synthase
Q830Z3	EF2621	Uncharacterized protein
Q834N3	EF1611	Probable manganese-dependent inorganic pyrophosphatase
Q834M9	EF1616	CoA-binding domain protein
Q839E0	EF0232	30S ribosomal protein S11
Q838R7	EF0368	Aspartokinase
Q836Q9	EF0149	6-phosphogluconate dehydrogenase, decarboxylating
Q839G1	EF0209	50S ribosomal protein L2
Q836D3	EF1182	S-ribosylhomocysteine lyase
Q831S5	EF2425	Phosphoglucomutase/phosphomannomutase family protein
Q834S3	EF1560	Uncharacterized protein
Q839H1	EF0198	30S ribosomal protein S12
Q837F1	EF0891	Aminotransferase
Q830Z2	EF2622	Uncharacterized protein
Q82ZJ2	EF3064	Polyribonucleotide nucleotidyltransferase
Q82Z22	EF3257	Oxidoreductase, pyridine nucleotide-disulfide family
Q838N8	EF0401	Pyrrolidone-carboxylate peptidase
Q835M1	EF1356	Dihydrolipoyl dehydrogenase
Q834H1	EF1681	Peptide methionine sulfoxide reductase MsrA
Q831A3	EF2610	ATP synthase subunit alpha
Q839I7	EF0178	ABC transporter, ATP-binding protein
Q835V2	EF1270	Ribosome maturation factor RimP
Q830B1	EF2882	Malonyl CoA-acyl carrier protein transacylase
Q834A7	EF1763	Protein translocase subunit SecA
Q830Y0	EF2638	Redox-sensing transcriptional repressor Rex 1
Q839I2	EF0185	Phosphopentomutase
P37710	EF0799	Autolysin

336

Q82Z74	EF3198	Lipoprotein, YaeC family	337
Q835J8	EF1379	Alanine--tRNA ligase	
Q831F9	EF2550	Serine hydroxymethyltransferase	338
Q820V4	EF0283	3-oxoacyl-[acyl-carrier-protein] synthase 2	
Q838J4	EF0453	OsmC/Ohr family protein	339
Q836Y7	EF0957	Glycosyl hydrolase, family 65	340
Q837E3	EF0900	Aldehyde-alcohol dehydrogenase	
Q831K3	EF2501	Arsenate reductase, putative	
Q837Y9	EF0685	Foldase protein PrsA	
Q839D9	EF0233	DNA-directed RNA polymerase subunit alpha	
Q834G9	EF1684	DegV family protein, putative	
P37061	EF1586	NADH oxidase	
Q835J3	EF1384	Phosphatidylglycerol lysyltransferase	
Q836R3	EF1045	ATP-dependent 6-phosphofructokinase	
Q839E8	EF0223	50S ribosomal protein L18	
Q831Y0	EF2365	Xanthine phosphoribosyltransferase	
Q831G0	EF2549	Uracil phosphoribosyltransferase	
Q830B2	EF2879	Acetyl-CoA carboxylase, biotin carboxyl carrier protein	
Q82Z92	EF3178	Peptidase, M20/M25/M40 family	
Q833K9	EF1938	Cation-transporting ATPase, E1-E2 family	
Q836J5	EF1116	Phenylalanine--tRNA ligase beta subunit	
Q836H2	EF1140	Lactoylglutathione lyase	
Q837W9	EF0706	ATP-dependent Clp protease, ATP-binding subunit ClpE	

341 Discussion

342 In this work we present significant progress in the study of H₂S as a gasotransmitter and
343 its link to antibiotic resistance.

344 *S. Typhimurium* is one of the classic H₂S producing bacterial species, via its *phsABC*
345 operon [18]. It is known that its capacity to reduce sulfur compounds provides
346 *Salmonella* with a growth advantage when colonizing the digestive tract [17,33].
347 However, the physiology and involvement of the *phsABC* operon in antibiotic
348 resistance, which has both a significant scientific and clinical interest, is not described.

349 The aim of this study was to understand the role and co-regulation of the H₂S-producing
350 pathways in *S. Typhimurium*, its possible role in microbial ecology and its effects on
351 other bacteria. By knocking-out the *phsABC* operon, we have found that the PHS
352 pathway is the predominant mechanism for H₂S production in this species. Other
353 pathways may or may not be active, but the lack of the PHS pathway is not
354 compensated by other mechanisms.

355 We have demonstrated that the *phsABC* operon is not involved in own antibiotic
356 resistance, even though it is the main source of H₂S in *S. Typhimurium*. This suggests
357 that the modifications in antibiotic resistance previously described in this species
358 [20,34] are not due to the H₂S itself. In fact, these authors state that the accumulation of
359 H₂S in their experiments is due to either a decrease in cysteine synthesis or an increase
360 in its catabolism. Cysteine is a well-known inducer of the Fenton reaction and the H₂S-
361 mediated augmentation of antibiotic resistance is associated with oxidative stress
362 protection [15]. This means that H₂S itself may be irrelevant for antibiotic resistance in
363 *S. Typhimurium*, which explains the absence of differences between WT and Δphs
364 strains regarding every aspect we have tested (except for the production of H₂S itself).

365 As we could not detect any intracellular role for H₂S in *S. Typhimurium*, but we had
366 verified that the *phsABC* operon was used to generate large amounts of H₂S, we
367 hypothesized that this H₂S may have an extracellular effect, that is, on neighboring
368 bacteria. Moreover, it is known that thiosulfate is abundant in the digestive tract [17],
369 suggesting that the *phsABC* operon should be active.

370 When *E. faecalis* was tested, instead of observing an enhancement of antibiotic
371 resistance by H₂S, as previously described [15,20], we observed that this gas is capable
372 of disrupting the intrinsic antibiotic resistance of *Enterococcus*. The MIC of cefotaxime
373 in *E. faecalis* was dramatically decreased and resistance to other cephalosporins was
374 similarly reverted, both in *E. faecalis* and *E. faecium* multiresistant enterococci,
375 responsible for endocarditis and septicemia in hospitals worldwide, are ranked by the
376 WHO among the pathogens for which effective therapies are critically needed [35]. In
377 addition, the use of certain antibiotics is a predisposing factor for suffering an
378 overgrowth and subsequent infections by *Enterococcus* [36,37]. Therefore, as
379 pharmacological donors of H₂S have already been developed [38–41], our work gives
380 new hope for the treatment of enterococcal infections. H₂S donor drugs could be
381 administered together with cephalosporins to effectively kill multidrug resistant
382 enterococci in patients. In addition, unraveling the mechanism of action of H₂S inside
383 *Enterococcus* could lead to the design of molecules that emulate H₂S function.

384 In any case, considering that the supply of new antibiotics to the global market is
385 virtually non-existent these days, the possibility of employing classical antibiotics that,
386 until now, were unavoidably discarded beforehand to treat multiresistant infections,
387 turns out encouraging in the fight against antibiotic resistance.

388

389 In our experiments, we observed that H₂S exhibits bactericidal synergy with a select
390 group of cephalosporins containing a methoxy-imino group in its structure. This residue
391 improves the drug's stability to β -lactamases and improves the ability of the drug to
392 cross the external membrane of gram-negative bacteria [42]. Clearly, these properties do
393 not account for its efficacy in the presence of H₂S, as β -lactamase production by
394 *Enterococcus* is anecdotic [43]. But, interestingly, many of the studies regarding
395 cephalosporin resistance in enterococci were obtained with this specific group of
396 methoxy-imino cephalosporins [44–49]. Thus, H₂S might be affecting some of the
397 pathways already described for *Enterococcus* cephalosporin resistance.

398 These pathways include a penicillin-binding protein that presents low affinity to these
399 drugs [50], the MurAA enzyme involved in peptidoglycan synthesis [47], mutations of
400 the β -subunit of the RNA polymerase [49], CroR/CroS and IreK/IreP two-component
401 systems [45,48], or alterations in the thymidylate synthesis pathway [44], among others.
402 Still, most of these studies do not unravel the final mechanisms governing
403 cephalosporin resistance and they suggest a common currently undescribed mechanism
404 [47,48,51]. Besides, in our experiments we have also observed an induction of
405 aminoglycosides susceptibility by H₂S (S3 Table), which it has not been observed by
406 other authors. Therefore, H₂S could be acting through a different pathway or upstream
407 of a common mechanism, a theory that would be also supported by the fact that no
408 known protein involved in cephalosporin resistance has been detected in the
409 persulfidome (Table 3). In any case, aminoglycosides constitute another group of last
410 resort antibiotics widely used in clinical settings and for which enterococci present a
411 modest level of intrinsic resistance [52]. It would be interesting to study in depth this
412 additional synergy between H₂S and aminoglycosides, although it has not been object of

413 study in this work, since the reversion of cephalosporin resistance in enterococci is
414 more relevant from a clinical and microbiological perspective.

415 From an ecological perspective, it is noteworthy that, in raw meat, enterococci have
416 been shown to successfully inhibit H₂S production by sulfide-producing bacteria,
417 presumably through enterocins or other biologically active compounds present in the
418 supernatant [21], although no further studies have addressed this phenomenon. We have
419 observed that, when exposed to H₂S, *Enterococcus* does not acquire additional
420 protection against antibiotics, but, in contrast, it is killed by a powerful group of
421 antimicrobial agents, to which this species demonstrates a robust resistance. Therefore,
422 this alteration of antibiotic resistance can explain why enterococci gain a selective
423 advantage by blocking the H₂S production of its neighbors. Enterococci constitute a
424 small proportion of the physiological microbiota of both humans and animals [53].
425 Thus, both *Salmonella* and *Enterococcus* are pathogens which pursue the same niche
426 colonization as part of their pathogenesis [17,37], which explains why both species
427 would benefit if the other one is ousted. In addition, we have demonstrated how other
428 H₂S producing species, including *Proteus vulgaris* (which also share the same
429 ecological niche), can also be involved in this interplay between bacteria.

430 Thus, this study highlights a new role for bacterial H₂S production. We show how
431 *Salmonella* and *Proteus*, through their H₂S production, can obtain an unexpected
432 advantage for niche competition (Fig 12). Aerial interaction among bacteria has been
433 previously revealed, but, to the best of our knowledge, this is the first report about H₂S
434 aerial communication, showing how a bacterium can exert an effect with lethal
435 consequences on another bacterium through H₂S, a gasotransmitter with vital functions
436 and abundant in the human body and to date considered a beneficial gas for bacteria.

437 **Acknowledgements**

438 We thank A. Hoefer for careful reading of the manuscript and G. Moyano for his assistance in
439 statistics. We thank Eugeny Nudler for providing us with the *E. coli* MG1655 strain.

440

441 **Competing interests**

442 The authors claim no conflict of interest.

443

444 **Funding**

445 The funders had no role in study design, data collection and analysis, decision to
446 publish, or preparation of the manuscript.

447 DTL and LC work have been supported by a FPU PhD scholarship from the Ministry of
448 Education of Spain (AP2012-2130 and AP2008-00586, respectively).

449 SM was funded by a FEMS long-term fellowship.

450 NM work is funded by the EvoTAR and EFFORT European projects.

451 European Commission (EC) EFFORT-613754-FP7 Bruno Gonzalez-Zorn

452 European Commission (EC) EVoTAR-282004-FP7 Bruno Gonzalez-Zorn

453 MRF acknowledges the support from ATIP-Avenir and “Investments for the future”

454 Programme IdEx Bordeaux, and the reference no. “ANR-10-IDEX-03-02”

455

456

457 **Authorship**

458 Conceptualization: DTL, LC, BGZ. Investigation: DTL, NM, SM. Methodology: DTL,
459 SM, SC, BGZ, MF. Project administration: BGZ. Supervision: BGZ, MF. Writing and
460 review: DTL, BGZ, LC, MF.

461

462 **Competing interests**

463 The corresponding author declares, on behalf of all authors, that there are no financial,
464 personal or professional interests that could be construed to have influenced the work.

465

466 Materials and Methods

467

468 Strains, media and reagents

469 All experiments were performed in Tryptone-Soy Agar or Broth (TSA or TSB,
470 respectively) purchased from Oxoid (Oxoid Ltd., UK), unless otherwise stated.
471 Antibiotic disks were acquired from bioMérieux (bioMérieux SA, Marcy l'Etoile,
472 France) and Oxoid. Antibiotic powder, sodium thiosulfate and other reagents were
473 purchased from Sigma (Sigma-Aldrich Química SA, Spain). NaHS was acquired from
474 Cayman (Cayman Chemical, USA).

475 The strains used in these experiments are listed in Table 4.

476

477 **Table 4. Bacteria used in this study.**

478

Strain	Abbreviated name	Description	Reference
<i>Enterococcus</i>			
<i>E. faecalis</i> VE14089	V583 WT	V583 strain cured from its plasmids	Rigottier-Gois <i>et al.</i> 2011
<i>E. faecalis</i> JH2-2	JH2-2 WT	Laboratory strain	Jacob & Hobbs 1974
<i>E. faecium</i> ATCC19434	<i>E. faecium</i>	Type strain	ATCC
<i>Salmonella</i> Typhimurium			
<i>S. enterica enterica</i> serovar Typhimurium ATCC14028 wild type	S. Typhimurium WT	Type strain	ATCC
<i>S. Typhimurium</i> $\Delta phsABC$ kan^R	kan^R	<i>phsABC</i> operon substituted with the kanamycin resistance cassette	This work
<i>S. Typhimurium</i> $\Delta phsABC$	Δphs	<i>phsABC</i> operon deletion mutant	This work
<i>S. Typhimurium</i> $\Delta phsABC$ (pTrc99a)	Δphs (pTrc99a)	Δphs mutant transformed with pTrc99a	This work
<i>S. Typhimurium</i> $\Delta phsABC$ (pH _s S)	Δphs^c	Δphs mutant transformed with pSB74	This work
<i>Escherichia coli</i>			
<i>E. coli</i> MG1655	<i>E. coli</i> WT	Laboratory strain	ATCC
<i>E. coli</i> MG1655 (pH _s S)	<i>E. coli</i> (pH _s S)	MG1655 strain transformed with pSB74	This work

Other strains

<i>Bacillus cereus</i> CECT193	<i>B. cereus</i>	Type strain	CECT
<i>Citrobacter freundii</i>	<i>C. freundii</i>	Strain I9	Our laboratory
<i>Enterobacter cloacae</i>	<i>E. cloacae</i>	Strain RyC: L15	Our laboratory
<i>Klebsiella pneumoniae</i> ATCC10031	<i>K. pneumoniae</i>	Type strain	ATCC
<i>Listeria ivanovii</i> ATCC19119	<i>L. ivanovii</i>	Type strain	ATCC
<i>Proteus vulgaris</i> CECT174	<i>P. vulgaris</i>	Type strain	CECT
<i>Pseudomonas aeruginosa</i>	<i>P. aeruginosa</i>	Strain A26	Our laboratory
<i>Staphylococcus aureus</i> COL	MRSA	Methicillin resistant <i>S. aureus</i>	Archer <i>et al.</i> 1994

479 **ATCC: American Type Culture Collection; CECT: Spanish Type Culture Collection.**

480

481 Primers used and DNA manipulation

482 Reactives used for PCR were purchased from Biotools (Biotools, Madrid, Spain). TAQ
483 polymerase (Biotools) or Phusion polymerase (Thermo Fisher Scientific Inc, USA) was
484 used. PCR products were purified with the PCR purification kit (Qiagen, In.c,
485 Chatsworth, CA). DNA was sequenced by Sanger (Secugen SL., Madrid, Spain).
486 Plasmids were extracted with the QIAprep Miniprep and Midiprep kits.

487 Primers used are listed in S5 Table. All primers were purchased from Sigma-Aldrich.

488

489 Plasmids used in this study

490 Plasmid pTrc99a was kindly donated by Professor Javier Turnay from the Biochemistry
491 Department of the Complutense University of Madrid. pSB74 [23] was acquired from
492 Addgene (plasmid #19591). Plasmid pKD13 (GenBank Accession number AY048744)
493 was used as a PCR template in order to construct the deletion mutants and it contains an
494 FLP recombination target (FRT)-flanked kanamycin resistance (*kan*) gene. Arabinose-
495 induced Red helper plasmid pKD46 (GenBank Accession number AY048746) was used

for the homologous recombination of the previously generated PCR products with the chromosome of *S. Typhimurium* ATCC14028. pCP20 FLP helper plasmid [54] was used for the elimination of the FRT-flanked resistance gene.

Plasmids used are listed in S6 Table.

Construction of *S. Typhimurium* mutants

The *S. Typhimurium phsABC* deletion mutant was constructed as previously described [55,56]. Briefly, a PCR was performed using pKD13 DNA as the for the amplification of the FRT flanked kanamycin resistance (*kan*) gene subsequently used for mutant selection (3). The 5'-terminal deletion primer had a 50-nucleotide (nt) homologous extension that includes the *phsA* initiation codon, and the 20-nt 5'-ATTCCGGGGATCCGTCGACC-3' priming site from pKD13. The 3'-terminal deletion primer consisted of a 50-nt homologous extension that includes 21 nt for the *phsC* C-terminal region, the termination codon and 29 nt downstream, and the 20-nt 5'-TGTAGGCTGGAGCTGCTTCG-3' priming site from pKD13. The PCR product obtained, containing the FRT flanked kanamycin resistance gene in between *phsA* and *phsC* homologous regions, was transformed in *S. Typhimurium* ATCC14028 carrying the thermosensitive arabinose-induced plasmid pKD46. Upon addition of arabinose, kanamycin resistant colonies (that is, those who have had their *phsABC* operon replaced by the kanamycin cassette) were selected. pCP20 FLP helper plasmid was used for the elimination of the FRT-flanked resistance gene from the $\Delta phsABC$ deletion mutants. Correct in-frame deletion of the operon was checked with the primers *phsABC_ext_F* and *phsABC_ext_R*.

520 **Antibiograms and Minimal Inhibitory Concentration determination**

521 Antibiograms were carried out in TSA media and interpreted following official
522 guidelines (CLSI 2017; EUCAST 2017). Minimal Inhibitory Concentrations (MIC) of
523 *E. faecalis* and *E. faecium* were determined by using E-test (bioMérieux) on TSA
524 media. MICs of *S. Typhimurium* were determined in TSB by the broth microdilution
525 method following official guidelines.

526

527 **Measurement of H₂S production**

528 Kligler media was purchased from bioMérieux. Tested strains freshly grown on TSA
529 were inoculated on Kligler media and incubated overnight (o.n.), with the cap both tight
530 and loose (to ensure aerobiosis conditions). Lead acetate paper strips were purchased
531 from Sigma-Aldrich and used according to manufacturer's instructions. Briefly, peptone
532 water (purchased from Oxoid) tubes were inoculated with the tested strains. Then,
533 between the cap and the inner wall of the tube we placed a strip, attached with adhesive
534 tape and above the inoculated medium, and the cap was slightly tight. Samples were
535 incubated for 18-24 hours at 37 °C.

536

537 **Confrontation experiments**

538 For the confronting experiments (Fig 2), a distance of 1 cm between both bacteria was
539 settled. To this end, 30 mL media plates were prepared, containing TSA, TSA
540 supplemented with sodium thiosulfate (T-S) 2 or 20 mM, or TSA supplemented with
541 cysteine (Cys) 2 mM. T-S and cysteine were prepared within the last seven days prior to
542 the experiment. First, simulating the preparation of an antibiogram, a 0.5 McFarland

543 turbidity suspension of the H₂S producing species (or its mutant) was spread evenly
544 over the entire surface of a TSA plate, supplemented with sodium thiosulfate T-S 2
545 mM, T-S 20 mM or Cys 2 mM prepared within the last seven days. Then, antibiograms
546 of the strains to be tested were prepared in TSA, attending to official guidelines. Finally,
547 lids were removed and both plates were fixed together with two pieces of adhesive tape,
548 facing each other. The edges zone was “sealed” with air permeable Parafilm M (Bemis
549 Company Inc, Oshkosh, WI). Plates were incubated at 37 °C for 24 hours maximum.
550 Anaerobiosis conditions were achieved by using the GENBag system from bioMérieux.

551

552 **Growth curves**

553 Growth curves of *S. Typhimurium* WT and its Δphs mutant were performed in Lennox
554 broth media (Conda Laboratorios, Spain), as published [15]. Briefly, from an o.n.
555 inoculum grown at 30 °C, a 1:100 dilution was performed in fresh media and samples
556 were grown at 37 °C to 0.7-0.8 optical density at 600 nm (O.D.). Afterwards, a 1:100
557 dilution was carried out in 10 mL and samples were incubated at 37 °C and 150 rpm,
558 measuring O.D. every hour. T-S 20 mM or different antibiotics were added when
559 indicated.

560

561 **Experiments incubated with H₂S**

562 For experiments in the presence of H₂S (Fig 6), *E. faecalis* antibiograms were prepared
563 in TSA plates following official guideline and were placed in a glass chamber of 2
564 liters volume. Next to them, we placed the base of an empty Petri dish in which we
565 positioned 0.1 grams of NaHS and, separated, 2 mL of ultra-pure water. Then, we

hermetically sealed the chamber and, only afterwards, NaHS and water were put in contact by gently shaking the chamber. Antibigrams were incubated for a maximum of 24 hours at 37 °C.

Time killing experiments

10 µL from an o.n. culture of *E. faecalis* in TSB were inoculated to 15 mL glass bottles containing 10 mL of TSB. When required, antibiotics at their proper concentration were added. Bottles were incubated at 37 °C and 100 rpm. Those bottles that required H₂S presence were placed in a glass hermetic chamber the same way as with experiments from the previous section (Fig 6). At 12 hours proper dilutions were plated for colony forming units (CFU) counting. From that moment on, all bottles were incubated under normal conditions until 24 hours, when dilutions were plated again.

Random transposition mutagenesis

The library of mutants was obtained following the protocol designed by Zhang and collaborators [59]. Plasmid pZXL5 was transformed in the strain *E. faecalis* JH2-2. This thermosensitive plasmid contains: ColE and pWV01 origins of replication (for gram negative and gram positive, respectively), a *mariner* transposon (containing the *aax(6')*-*apg(2'')* gene for gentamicin resistance within), a nisin-inducible *mariner* transposase (with the system *nisK-nisR* and a chloramphenicol resistance cassette (*cat*)).

Bacteria containing pZXL5 were incubated o.n. at 28 °C in BHI media containing gentamicin 300 mg/L and chloramphenicol 10 mg/L. Afterwards, a 1:2000 dilution was carried out in fresh BHI media containing 300 mg/L and nisin 0.025 mg/L to induce the

589 random insertion of the transposon in the genome. Then, incubation of the samples at
590 150 rpm and 37 °C in the presence of gentamicin allows the specific selection of the
591 bacteria possessing the gentamicin resistance gene (and, therefore, the transposon) in
592 the chromosome.

593 Afterwards, a 1:2000 dilution in fresh media containing gentamicin was performed and
594 bacteria were incubated at 37 °C and 150 rpm o.n., obtaining a library of mutants that
595 was stored at -80 °C for future experiments.

596 By incubating in the presence of gentamicin, cefotaxime and H₂S, we attempted to
597 select only those mutants that resisted the combined action of H₂S and cefotaxime,
598 presumably because the transposon would have interrupted a pathway key in the
599 susceptibilization of *Enterococci*.

600

601 **BTA method for persulfide detection**

602 Bottles containing fresh TSB or TSB-CTX 8 µg/mL were inoculated with an o.n.
603 inoculum of *E. faecalis* JH2-2, in a 1:1000 proportion. Samples were incubated for 5.5
604 hours at 37 °C and 100 rpm. Then, each sample was split in two and, to one of each set,
605 H₂S 250 µM (freshly prepared as previously described) was added. The four bottles
606 were incubated for 30 minutes at 37 °C and 100 rpm. Then, samples were centrifuged
607 for 3 minutes at 6000 rpm and supernatant was discarded. Samples were washed once
608 with PBS, centrifuged and supernatant discarded again. Then, each sample was mixed
609 with 1 mL of lysis buffer, containing HENP buffer (pH 7.41), SDS 1%, 10 µL of
610 protease inhibitor 1% and 100 µM of biotin maleimide. To ensure complete lysis,
611 samples were sonicated (3 cycles of 1 minute at 190 MHz and 2 minutes of pause).
612 Then, samples were incubated for 60 minutes at 37 °C.

613 After incubation, 20 mM of freshly prepared N-ethylmaleimide (NEM) was added to
614 block the unreacted cysteines and the samples were incubated o.n. at room T^a with
615 rotating shaking. During all the procedure the lysis buffer and the samples were
616 protected from light.

617 Proteins were precipitated with CHCl₃/MeOH/H₂O precipitation (1/4/4, v/v), washed
618 twice and resuspended in HEPES buffer pH 7.4 containing SDS up to 0.5%. Samples
619 were adjusted to equal protein concentration. Biotinylated proteins were bound to
620 streptavidin agarose beads, and persulfidated targets eluted by DTT 0.5 mM (Fig 11).

621 Eluted proteins were either resolved by SDS electrophoresis and visualized with silver
622 staining or subjected to in-gel trypsinization and subsequent LC/MS analysis.
623 Experiments were performed in triplicates.

624

625 **nanoLC/MS**

626 Protein samples were run on SDS-PAGE (10 %) but protein separation was stopped
627 once proteins have entered the resolving gel. After colloidal blue staining, each lane was
628 was cut into 1 mm x 1 mm gel pieces. The gel pieces were destained in 25 mM
629 ammonium bicarbonate 50% ACN, rinsed twice in ultrapure water and shrunk in ACN
630 for 10 min. After ACN removal, gel pieces were dried at room temperature,
631 carbamidomethylated and covered with the trypsin solution (10 ng/μL in 50 mM
632 NH₄HCO₃), rehydrated at 4°C for 10 min, and finally incubated overnight at 37°C. The
633 gel pieces were then incubated for 15 min in 50 mM NH₄HCO₃ at room temperature
634 with rotary shaking. The supernatant was collected, and an H₂O/ACN/HCOOH
635 (47.5:47.5:5) extraction solution was added onto the gel pieces for 15 min. The
636 extraction step was repeated twice. The pooled supernatants were dried in a vacuum

centrifuge and then resuspended in 40 μ L of water acidified with 0.1% HCOOH. Peptide mixture was analyzed on an Ultimate 3000 nanoLC system (Dionex, Amsterdam, The Netherlands) coupled to an Electrospray Q-Exactive quadrupole Orbitrap benchtop mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Ten microliters of peptide digests were loaded onto a 300- μ m-inner diameter x 5-mm C18 PepMapTM trap column (LC Packings) at a flow rate of 30 μ L/min. The peptides were eluted from the trap column onto an analytical 75-mm id x 15-cm C18 Pep-Map column (LC Packings) with a 4–40% linear gradient of solvent B in 108 min. Mobile phases were a mix of solvent A (0.1% formic acid in 5% ACN) and solvent B (0.1% formic acid in 80% ACN). The separation flow rate was set at 300 nL/min. The mass spectrometer operated in positive ion mode at a 1.9-kV needle voltage. Data were acquired using Xcalibur 2.2 software in a data-dependent mode. MS scans (m/z 300–2000) were recorded at a resolution of $R = 70000$ (@ m/z 200) and an automatic gain control (AGC) target of 3×10^6 ions collected within 100 ms. Dynamic exclusion was set to 30 s and the top 12 ions were selected from fragmentation in higher-energy collisional dissociation (HCD) mode. MS/MS scans with a target value of 1×10^5 ions were collected with a maximum fill time of 120 ms and a resolution of $R = 35000$. Additionally, only +2 and +3 charged ions were selected for fragmentation. Other settings were as follows: neither sheath nor auxiliary gas flow; heated capillary temperature, 270°C; normalized HCD collision energy of 27% and an isolation width of 2 m/z .

Database search and results processing

Data were searched by SEQUEST through Proteome Discoverer 1.4 (Thermo Fisher Scientific Inc.) against a subset of the 2017-03 version of UniProt database restricted to

662 *Enterococcus faecalis* Reference Proteome Set (3240 entries). Spectra from peptides
 663 higher than 5000 Da or lower than 350 Da were rejected. The search parameters were as
 664 follows: mass accuracy of the monoisotopic peptide precursor and peptide fragments
 665 was set to 10 ppm and 0.02 Da respectively. Only b- and y-ions were considered for
 666 mass calculation. Oxidation of methionines (+16 Da) was considered as variable
 667 modification and carbamidomethylation of cysteines (+57 Da) as fixed modification.
 668 Two missed trypsin cleavages were allowed. Peptide validation was performed using
 669 Percolator algorithm [60] and only “high confidence” peptides were retained
 670 corresponding to a 1% False Positive Rate at peptide level.
 671

672 **Statistical analysis**

673 The statistical analysis for this paper was generated using SAS software Pre-production
 674 version 9.0 (SAS Institute Inc., Cary, NC, USA).

675

676 **Figure captions**

677 **Fig 1. Characterization of the *S. Typhimurium phsABC* operon.**

678 (A) PCR showing substitution of the operon by the kanamycin cassette, and subsequent deletion
679 of this cassette. (B) H₂S production, measured in Kligler media in the absence (upper line) or
680 the presence (bottom line) of thiosulfate 20 mM (C) or in peptone water broth by lead acetate
681 strips.

682

683 **Fig 2. Confronting experiments.**

684 An antibiogram of the species to be tested is exposed to another plate streaked with an H₂S
685 producing species and supplemented with thiosulfate 20 mM (T-S).

686

687 **Fig 3. Confronting experiments of *E. faecalis*.**

688 Control antibiograms and confronting experiments of *E. faecalis* JH2-2 (plated on TSA media)
689 faced with *S. Typhimurium* ATCC14028, *E. coli* (pH₂S), *Proteus vulgaris* CECT174. H₂S
690 producing species were plated on TSA media containing T-S 20 mM. Antibiotics tested:
691 cloramphenicol (C), vancomycin (VAN), tetracycline (TET), sulphametoxazol-trimethoprim
692 (SXT), clindamycin (CMN), rifampin (RA), ciprofloxacin (CIP), streptomycin (S), nalidixic
693 acid (NA), gentamicin (GMN), amoxicillin (AMX), aztreonam (ATM), cefotaxime (CTX),
694 cefuroxime (CXM), ertapenem (ETP), cefotetan (CTT). Antibiotics showing synergy with H₂S
695 are highlighted.

696

697 **Fig 4. Minimal Inhibitory Concentration of *E. faecalis* confronted with *S. Typhimurium*.**

698 MIC for cefotaxime (TX) of *E. faecalis* JH2-2 control strain (left), confronted with *S.*
699 Typhimurium WT (center) and with *S. Typhimurium* Δphs (right).

700

701 **Fig 5. H₂S production by *E. coli* WT and *E. coli* p(H₂S).**

702 H₂S production was measured with Kligler media (left) and lead acetate strips (right).

703

704 **Fig 6. Method for incubation with chemical H₂S.**

705 Samples were placed in a chamber, and next to it the NaHS stone (represented as a star) was
706 placed on an empty Petri dish alongside 2 mL of ultrapure water (dashed blue area); after
707 sealing the chamber, it is gently shaken to release the H₂S to the atmosphere upon contact with
708 H₂O (B). CTX: cefotaxime.

709

710 **Fig 7. Antibigrams of *E. faecalis* in the presence of H₂S.**

711 *E. faecalis* V583 was incubated in a chamber in the presence and absence of NaHS 0.1 grams,
712 for the antibiotics cefotaxime (CTX), cefuroxime (CXM), ceftizoxime (ZOX), cefotetan (CTT)
713 and cefpodoxime (CPD).

714

715 **Fig 8. Antibigrams of *E. faecium* in the presence of H₂S.**

716 *E. faecium* ATCC19434 was incubated in a chamber in the absence (left) and the presence
717 (right) of 0.1 grams of NaHS, for the antibiotics cefotaxime (CTX), cefuroxime (CXM),
718 ceftizoxime (ZOX), cefotetan (CTT) and cefpodoxime (CPD).

719

720 **Fig 9. Chemical structure of cephalosporins.**

721 In cefotaxime the methoxy-imino residue is emphasized.

722

723 **Fig 10. *E. faecalis* time killing curves.**

724 *E. faecalis* JH2-2 strain was incubated in TSB liquid media in the presence and absence of
725 NaHS (in the atmosphere) and/or cefotaxime (CTX). Dilutions were plated at 0, 12 and 24 hours
726 for Colony Forming Units (CFU) counting. Error bars represent the standard error of at least
727 three independent experiments. Significant differences among samples are indicated as a, b, c, d.
728 Samples with the same letter are not statistically different among them.

729

730 **Fig 11. Identification of the complete persulfidome of *E. faecalis***

731 (A) Workflow used for the specific labeling of persulfide residues. Biotin maleimide binds only
732 to thiol and persulfide motifs. Bound proteins are separated from others by streptavidin
733 magnetic beads. DTT treatment specifically cleaves disulfide bridges with biotin maleimide,
734 rendering the original S-sulfhydrated proteins available for subsequent procedures. (B) SDS-
735 PAGE of *E. faecalis* total protein extraction incubated in the presence and absence of H₂S and
736 CTX.

737

738 **Fig 12. Niche competition of *Enterococcus* and H₂S-producing bacteria**

739 The use of cephalosporins or other antibiotics to which *E. faecalis* and *E. faecium* are resistant
740 promotes these species overgrowth in the host's digestive tract, facilitating their dissemination
741 and the development of enterococcal infections (upper panel). Nevertheless, a cephalosporin-
742 resistant *S. Typhimurium* strain in the niche could induce the killing of enterococci by H₂S
743 production, displacing the latter and colonizing the gut as a previous step for infection (bottom
744 panel).

745

746

747

748

749 **References**

- 750 1. Papenfort K, Bassler BL. Quorum sensing signal-response systems in Gram-negative bacteria. *Nat Rev*
751 *Micro.* Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2016
752 Sep;14(9):576–88.
- 753 2. Heal RD, Parsons AT. Novel intercellular communication system in *Escherichia coli* that confers antibiotic
754 resistance between physically separated populations. *J Appl Microbiol.* 2002;92(6):1116–22.
- 755 3. Bernier SP, Létoffé S, Delepierre M, Ghigo JM. Biogenic ammonia modifies antibiotic resistance at a
756 distance in physically separated bacteria. *Mol Microbiol.* 2011;81(3):705–16.
- 757 4. Létoffé S, Audrain B, Bernier SP, Delepierre M, Ghigo J. Aerial Exposure to the Bacterial Volatile
758 Compound Trimethylamine Modifies Antibiotic Resistance of Physically Separated Bacteria by.
759 2014;5(1):1–12.
- 760 5. Chen Y, Gozz K, Yan F, Chai Y. Acetic acid acts as a volatile signal to stimulate bacterial biofilm
761 formation. *MBio.* 2015;6(3):1–13.
- 762 6. Wang RUI. Two 's company , three ' s a crowd: can H₂S be the third endogenous gaseous transmitter?
763 *Faseb J.* 2002;16(13):1792–8.
- 764 7. Attene-Ramos MS. Evidence That Hydrogen Sulfide Is a Genotoxic Agent. *Mol Cancer Res.* 2006;4(1):9–
765 14.
- 766 8. Ryter SW, Morse D, Choi AMK. Carbon Monoxide: To Boldly Go Where NO Has Gone Before. *Science*
767 (80-). 2004;27(April):208–33.
- 768 9. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, et al. H₂S as a physiologic vasorelaxant: hypertension in
769 mice with deletion of cystathionine gamma-lyase. *Science.* 2008;322(5901):587–90.
- 770 10. Li Q, Lancaster JR. Chemical foundations of hydrogen sulfide biology. *Nitric Oxide.* Elsevier Inc.;
771 2013;35(July):21–34.
- 772 11. Zhao K, Ju Y, Li S, Altaany Z, Wang R, Yang G. S-sulphydration of MEK 1 leads to PARP-1 activation and
773 DNA damage repair. 2014;15(7):792–800.
- 774 12. Revsbech IG, Shen X, Chakravarti R, Jensen FB, Thiel B, Evans AL, et al. Hydrogen sulfide and nitric oxide
775 metabolites in the blood of free-ranging brown bears and their potential roles in hibernation. *Free Radic Biol*
776 *Med.* Elsevier; 2014;73:349–57.
- 777 13. Ni Z-J, Hu K-D, Song C-B, Ma R-H, Li Z-R, Zheng J-L, et al. Hydrogen Sulfide Alleviates Postharvest
778 Senescence of Grape by Modulating the Antioxidant Defenses. *Oxid Med Cell Longev.* 2016;2016:1–14.
- 779 14. Gusarov I, Shatalin K, Starodubtseva M, Nudler E. Endogenous Nitric Oxide Protects Bacteria Against a
780 Wide Spectrum of Antibiotics. *Science* (80-). 2009;325(5946):1380–4.
- 781 15. Shatalin K, Shatalina E, Mironov A, Nudler E. H₂S: a universal defense against antibiotics in bacteria.

782 Science (80-). 2011;334(6058):986–90.

783 16. Clarke PH. Hydrogen Sulphide Production by Bacteria. J Gen Microbiol. 1953;8(3):397–407.

784 17. Winter SE, Thiennimitr P, Winter MG, Butler BP, Huseby DL, Crawford RW, et al. Gut inflammation
785 provides a respiratory electron acceptor for *Salmonella*. Nature. 2010;467(7314):426–9.

786 18. Heinzinger NK, Fujimoto SY, Clark MA, Moreno MS, Barrett EL. Sequence analysis of the phs operon in
787 *Salmonella typhimurium* and the contribution of thiosulfate reduction to anaerobic energy metabolism. J
788 Bacteriol. 1995;177(10):2813–20.

789 19. Álvarez R, Frávega J, Rodas PI, Fuentes JA, Paredes-Sabja D, Calderón IL, et al. Participation of S.
790 Typhimurium cysJIH Operon in the H₂S-mediated Ciprofloxacin Resistance in Presence of Sulfate as Sulfur
791 Source. Antibiotics. 2015;4(3):321–8.

792 20. Frávega J, Álvarez R, Díaz F, Inostroza O, Tejías C, Rodas PI, et al. *Salmonella Typhimurium* exhibits
793 fluoroquinolone resistance mediated by the accumulation of the antioxidant molecule H₂S in a CysK-
794 dependent manner. J Antimicrob Chemother. 2016;

795 21. Liu X, Gong C, Jiang X. Inhibitory effects of enterococci on the production of hydrogen sulfide by hydrogen
796 sulfide-producing bacteria in raw meat. J Appl Microbiol. 2011;111(1):83–92.

797 22. Barrett EL, Clark MA. Tetrathionate reduction and production of hydrogen sulfide from thiosulfate.
798 Microbiol Rev. 1987;51(2):192–205.

799 23. Bang SW, Clark DS, Keasling JD. Engineering hydrogen sulfide production and cadmium removal by
800 expression of the thiosulfate reductase gene (phsABC) from *Salmonella enterica* serovar typhimurium in
801 *Escherichia coli*. Appl Environ Microbiol. 2000;66(9):3939–44.

802 24. Wu F, Xu X, Xie J, Yi S, Wang J, Yang X, et al. Molecular characterization of *Salmonella enterica* serovar
803 Aberdeen negative for H₂S production in China. PLoS One. 2016;11(8):1–11.

804 25. Módos K, Panopoulos P, Coletta C, Papapetropoulos A, Szabo C. Hydrogen sulfide-mediated stimulation of
805 mitochondrial electron transport involves inhibition of the mitochondrial phosphodiesterase 2A, elevation of
806 cAMP and activation of protein kinase A. Biochem Pharmacol. 2013;86(9):1311–9.

807 26. Rice LB. Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No
808 ESKAPE. J Infect Dis. 2008;197(8):1079–81.

809 27. Zhang D, Macinkovic I, Devarie-Baez NO, Pan J, Park CM, Carroll KS, et al. Detection of protein S-
810 sulfhydration by a tag-switch technique. Angew Chemie (International ed.in English). Department of
811 Chemistry, Washington State University, Pullman, WA 99164 (USA).: WILEY-VCH Verlag GmbH & Co.
812 KGaA, Weinheim; 2014;53(2):575–81.

813 28. Moore PK, Whiteman M, Moore PK. Chemistry, Biochemistry and Pharmacology of Hydrogen Sulfide. Vol.
814 230, Hand. Exp. Pharmacol. 2015. 395 p.

815 29. Paul BD, Snyder SH. H₂S: A Novel Gasotransmitter that Signals by Sulfhydration. Trends Biochem Sci.
816 Elsevier Ltd; 2015;xx:1–14.

- 817 30. Wedmann R, Onderka C, Wei S, András Szijártó I, Miljkovic J, Femic A, et al. Improved Tag-Switch
818 Method Reveals that Thioredoxin Acts as Depersulfidase and Controls the Intracellular Levels of Protein
819 Persulfidation. *Chem Sci*. 2016;3414–26.
- 820 31. Cuevasanta E, Lange M, Bonanata J, Coitiño EL, Ferrer-Sueta G, Filipovic MR, et al. Reaction of hydrogen
821 sulfide with disulfide and Sulfenic acid to form the strongly Nucleophilic Persulfide. *J Biol Chem*.
822 2015;290(45):26866–80.
- 823 32. Gao XH, Krokowski D, Guan BJ, Bederman I, Majumder M, Parisien M, et al. Quantitative H₂S-mediated
824 protein sulphydration reveals metabolic reprogramming during the integrated stress response. *Elife*.
825 2015;4(NOVEMBER2015):1–21.
- 826 33. Bäumlér AJ, Sperandio V. Interactions between the microbiota and pathogenic bacteria in the gut. *Nature*.
827 2016;535(7610):85–93.
- 828 34. Álvarez R, Neumann G, Frávega J, Díaz F, Tejías C, Collao B, et al. CysB-dependent upregulation of the
829 *Salmonella Typhimurium* cysJIH operon in response to antimicrobial compounds that induce oxidative
830 stress. *Biochem Biophys Res Commun*. 2015;458(1):46–51.
- 831 35. Tacconelli E, Magrini N, Carmeli Y, Harbarth S, Kahlmeter G, Kluytmans J, et al. Global priority list of
832 antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *World Heal*
833 *Organ*. 2017;
- 834 36. Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, et al. Vancomycin-resistant *Enterococcus*
835 domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream
836 invasion in humans. *J Clin Invest*. Infectious Diseases Service, Department of Medicine, Memorial Sloan-
837 Kettering Cancer Center, New York, New York, USA. ubedamoc@mskcc.org; 2010;120(12):4332–41.
- 838 37. Hendrickx APA, Top J, Bayjanov JR, Kemperman H, Rogers MRC, Paganelli FL, et al. Antibiotic-driven
839 dysbiosis mediates intraluminal agglutination and alternative segregation of *enterococcus faecium* from the
840 intestinal epithelium. *MBio*. 2015;6(6):1–11.
- 841 38. Li L, Whiteman M, Guan YY, Neo KL, Cheng Y, Lee SW, et al. Characterization of a novel, water-soluble
842 hydrogen sulfide-releasing molecule (GYY4137): new insights into the biology of hydrogen sulfide.
843 *Circulation*. Department of Pharmacology, National University of Singapore, Singapore; 2008 May
844 6;117(18):2351–60.
- 845 39. Ahmad A, Olah G, Szczesny B, Wood ME, Whiteman M, Szabo C. AP39, a mitochondrially targeted
846 hydrogen sulfide donor, exerts protective effects in a rat model of renal ischemia–reperfusion. *Nitric Oxide*.
847 2015;47(2015):S43.
- 848 40. Gero D, Torregrossa R, Perry A, Waters A, Le-Trionnaire S, Whatmore JL, et al. The novel mitochondria-
849 targeted hydrogen sulfide (H₂S) donors AP123 and AP39 protect against hyperglycemic injury in
850 microvascular endothelial cells in vitro. *Pharmacol Res*. 2016;113:186–98.
- 851 41. Nußbaum BL, Vogt J, Wachter U, McCook O, Wepler M, Matallo J, et al. Metabolic, cardiac and renal
852 effects of the slow hydrogen sulfide-releasing molecule GYY4137 during resuscitated septic shock in swine
853 with pre-existing coronary artery disease. *Shock*. 2017;33(5):519–25.

- 854 42. Nikaido H, Liu W, Rosenberg EY. Outer membrane permeability and β -lactamase stability of dipolar ionic
855 cephalosporins containing methoxyimino substituents. *Antimicrob Agents Chemother.* 1990;34(2):337–42.
- 856 43. ECDC. Antimicrobial Resistance surveillance in Europe. 2015. 9-32 p.
- 857 44. Hoff JS, Kristich CJ. Thymidylate Limitation Potentiates Cephalosporin Activity toward Enterococci via an
858 Exopolysaccharide-Based Mechanism. *ACS Chem Biol.* 2016;11(6):1561–8.
- 859 45. Kristich CJ, Little JL, Hall CL, Hoff JS. Reciprocal regulation of cephalosporin resistance in *Enterococcus*
860 *faecalis*. *MBio.* Department of Microbiology and Molecular Genetics, Center for Infectious Disease
861 Research, Medical College of Wisconsin, Milwaukee, Wisconsin, USA. ckristich@mcw.edu; 2011 Nov
862 1;2(6):e00199-11.
- 863 46. Hall CL, Tschannen M, Worthey EA, Kristich CJ. IreB, a Ser/Thr kinase substrate, influences antimicrobial
864 resistance in *Enterococcus faecalis*. *Antimicrob Agents Chemother.* 2013;57(12):6179–86.
- 865 47. Vesic D, Kristich CJ. MurAA is required for intrinsic cephalosporin resistance of *Enterococcus faecalis*.
866 *Antimicrob Agents Chemother.* Department of Microbiology and Molecular Genetics, Center for Infectious
867 Disease Research, Medical College of Wisconsin, Milwaukee, Wisconsin, USA.; 2012 May;56(5):2443–51.
- 868 48. Kellogg SL, Kristich CJ. Functional dissection of the CroRS two-component system required for resistance
869 to cell wall stressors in *Enterococcus faecalis*. *J Bacteriol.* 2016;198(8):1326–36.
- 870 49. Kristich CJ, Little JL. Mutations in the beta subunit of RNA polymerase alter intrinsic cephalosporin
871 resistance in Enterococci. *Antimicrob Agents Chemother.* Department of Microbiology and Molecular
872 Genetics, Center for Infectious Disease Research, Medical College of Wisconsin, Milwaukee, Wisconsin,
873 USA. ckristich@mcw.edu; 2012;56(4):2022–7.
- 874 50. Sifaoui F, Arthur M, Rice L, Gutmann L. Role of penicillin-binding protein 5 in expression of ampicillin
875 resistance and peptidoglycan structure in *Enterococcus faecium*. *Antimicrob Agents Chemother.* L.R.M.A.,
876 INSERM E0004, Universite Paris VI, 75270 Paris Cedex 06, France.; 2001 Sep;45(9):2594–7.
- 877 51. Desbonnet C, Tait-Kamradt A, Garcia-Solache M, Dunman P, Coleman J, Arthur M, et al. Involvement of
878 the eukaryote-like kinase-phosphatase system and a protein that interacts with penicillin-binding protein 5 in
879 emergence of cephalosporin resistance in cephalosporin-sensitive class A penicillin-binding protein mutants
880 in *Enterococcus faeci*. *MBio.* 2016;7(2):1–10.
- 881 52. Hollenbeck BL, Rice LB. Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence.*
882 2012;3(5):421–33.
- 883 53. Svec P, Devriese L. *Enterococcus*. In: *Bergey's Manual of Systematics of Archaea and Bacteria.* Bergey's
884 Manual Trust; 2015. p. 1–25.
- 885 54. Cherepanov PP, Wackernagel W. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the
886 option of F⁺-catalyzed excision of the antibiotic-resistance determinant. *Gene.* 1995;158(1):9–14.
- 887 55. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR
888 products. *Proc Natl Acad Sci U S A.* Department of Biological Sciences, Purdue University, West Lafayette,
889 IN 47907, USA.; 2000 Jun 6;97(12):6640–5.

890 56. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of Escherichia coli K-12 in-
891 frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol. Institute for Advanced
892 Biosciences, Keio University, Tsuruoka City, Yamagata, Japan.; 2006;2:2006.0008.

893 57. [CLSI] Clinical Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility
894 Testing. 27th ed. CLSI supplement M100. 27th ed. Vol. fifteenth. 2017. 1-256 p.

895 58. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of
896 MICs and zone diameters. Version 7.0. 2017.

897 59. Zhang X, Paganelli FL, Bierschenk D, Kuipers A, Bonten MJ, Willems RJ, et al. Genome-wide
898 identification of ampicillin resistance determinants in Enterococcus faecium. PLoS Genet. Department of
899 Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands.; 2012
900 Jun;8(6):e1002804.

901 60. Käll L, Canterbury JD, Weston J, Noble WS, MacCoss MJ. Semi-supervised learning for peptide
902 identification from shotgun proteomics datasets. Nat Methods. 2007;4(11):923–5.

903

904

905 **Supplementary material**

906

907 **S1 Fig. *S. Typhimurium* WT and *Δphs* growth curves.**

908 Experiments were carried out both in the presence and absence of thiosulfate (T-S) 20 mM (A) and in the
 909 presence of ampicillin 0.5 mg/L (B). The error bars represent the standard error of three independent
 910 experiments. Minimal Inhibitory Concentrations of different antibiotics (C). The results show the mean of
 911 three independent experiments.

912

913 **S1 Table. Antibigrams of *Salmonella* Typhimurium WT and Δphs strain, in Tryptone Soy Agar**
 914 **(TSA), in the presence and absence of thiosulfate 20 mM (T-S).**

915

916		<i>S. Typhimurium</i> WT		<i>S. Typhimurium</i> Δphs	
		TSA	TSA + T-S 20	TSA	TSA + T-S 20
917	Amox. + Clavulanic acid 30	31	28	32	29
	Cefaclor 30	31	30	29	31
	Cefotaxime 30	34	34	34	34
918	Cefpodoxime 10	28	29	30	27
919	Cefoxitin 30	26	25	29	25
	Imipenem 10	33	32	33	32
920	Aztreonam 30	38	34	34	34
921	Apramycin 15	13	10	13	9
	Gentamicin 10	16	14	17	14
922	Neomycin 30	16	14	15	13
923	Netilmicin 30	19	17	19	17
	Estreptomycin 10	12	7	12	7
924	Ciprofloxacin 10	35	37	35	36
925	Sulpha - Trimetoprim 25	29	30	29	30
	Tetracycline 30	27	27	26	27
926	Clindamycin 2	6	6	6	6
927	Colistin 10	14	14	13	14
	Cloramphenicol 50	25	27	28	28
928	Erythromycin 15	7	6	6	7
929	Mupirocin 5	6	6	6	6
	Rifampin 5	9	7	9	9
930	Vancomycin 30	6	6	6	6
931	Fosfomycin 200	36	30	33	31

932

933

934

935

936

937

938

939

940

941

942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974

S2 Table. Minimal Inhibitory Concentration (MIC) for different antibiotics in *S. Typhimurium* WT and its *Aphs* mutant.

MIC (mg/L)	<i>S. Typhimurium</i> WT	<i>S. Typhimurium</i> <i>Aphs</i>
Ampicillin	1	1
Cefotaxime	0.125	0.062
Streptomycin	128	64
Cloramphenicol	8	8
Ciprofloxacin	0.031	0.031

975

976 **S3 Table. Antibigrams of *E. faecalis* V583 strain, in Tryptone Soy Agar (TSA), in the presence and**
 977 **absence of NaHS 0.10 grams.**

	TSA	TSA +H ₂ S 0.10 g
Ampicillin 10	28	>40
Amoxicillin 25	33	>40
Amox. + clavulanic acid 30	32	>40
Penicillin 10	24	30
Cefaclor 30	10	12
Cefotaxime 30	6	23
Ertapenem 10	6	6
Imipenem 10	29	37
Aztreonam 30	6	6
Amikacin 30	11	28
Apramycin 15	6	23
Streptomycin 10	6	24
Gentamicin 10	13	27
Kanamycin 30	11	28
Neomycin 30	10	24
Tobramycin 10	11	28
Nalidixic acid 30	6	6
Norfloxacin 10	23	25
Sulfamethoxazol 300	6	6
Sulfa - Trimetoprim 25	33	34
Tetracyclin 30	30	33
Clindamycin 2	6	6
Cloramphenicol 50	29	30
Colistin 10	6	6
Eritromycin 15	23	29
Fusidic acid 5	13	19
Lincomycin 15	7	9
Mupirocin 5	8	11
Pristinamycin 15	17	18
Rifampin 5	18	19
Vancomycin 30	15	19

978

979

980

981

982

983 **S4 Table. Tukey-Kramer post-hoc analysis results. Statistically significant results of interest are**
984 **highlighted.**

TUKEY-KRAMER POST-HOC ANALYSIS												
	WT 0	WT 12	WT 24	H ₂ S 0	H ₂ S 12	H ₂ S 24	CTX 0	CTX 12	CTX 24	H ₂ S- CTX 0	H ₂ S- CTX 12	H ₂ S- CTX 24
0		<u><.0001</u>	<u><.0001</u>	1.0000	<.0001	<.0001	1.0000	<.0001	<.0001	1.0000	0.0081	0.0004
WT 12			1.0000	<.0001	0.9257	0.9560	<.0001	<u>0.0188</u>	0.0137	<.0001	<u><.0001</u>	<.0001
24				<.0001	0.9984	0.9996	<.0001	0.0670	0.0629	<.0001	<.0001	<u><.0001</u>
0					<u><.0001</u>	<u><.0001</u>	1.0000	<.0001	<.0001	1.0000	0.0124	0.0006
H ₂ S 12						1.0000	<.0001	0.3500	0.3149	<.0001	<u><.0001</u>	<.0001
24							<.0001	0.2910	0.2520	<.0001	<.0001	<u><.0001</u>
0								<u><.0001</u>	<u><.0001</u>	1.0000	0.0206	0.0010
CTX 12									1.0000	<.0001	<u><.0001</u>	<.0001
24										<.0001	<.0001	<u><.0001</u>
0											<u>0.0142</u>	<u>0.0007</u>
H ₂ S - CTX 12												0.9914
24												

985

986

987

988

989

990

991

992

993

994

995

996

997 **S5 Table. Primers used in this study.**

Primer name	Sequence (5' → 3')	Source
H1_phsABC_P1	TACAAATTAAATATAATCTTCAGCTATATCTAATAAC AGGAGGTTATATGATTCCGGGGATCCGTGACC	This work
H2_phsABC_P2	TTCTTGCTGACATTATTTTATGGATACGCTCAGACCG CGGACTTATCCCCGCGTGTAGGCTGGAGCTGCTTCG	This work
phsABC_ext_F	GGTTTATCGTGTTGACATGC	This work
phsABC_ext_R	GTCAGGATGGTCTCTATTTGC	This work

998

999

1000

1001

1002

1003

1004

1005

1006

1007

1008

1009

1010

1011

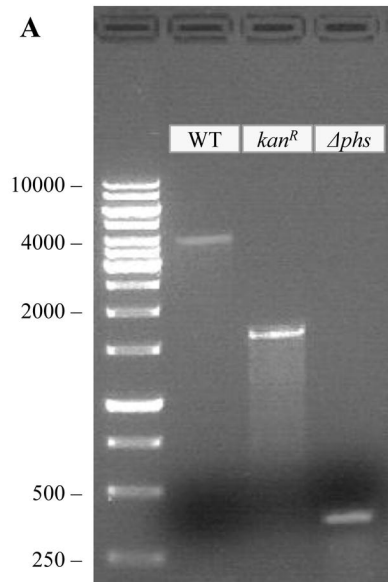
1012

1013 **S6 Table. Plasmids used in this study.**

Plasmid name	Description	GenBank Access number	Reference
<u>In-frame genes deletion</u>			
pKD46	Thermosensitive helper plasmid. It carries the λ phage Red recombination system. Ara ^{Ind} , Amp ^R	AY048746	Datsenko & Wanner 2000
pKD13	Kanamycin resistance cassette carrier plasmid.	AY048744	Datsenko & Wanner 2000
pCP20	Thermosensitive helper plasmid. It carries the FLP recombinase. Amp ^R	-	Cherepanov & Wackernagel 1995
<u>H₂S production</u>			
pTrc99a	Empty vector		Bang <i>et al.</i> 2000
pSB74 (pH ₂ S)	pTrc99a + <i>phsABC</i>	-	Bang <i>et al.</i> 2000
<u>Random transposition mutagenesis</u>			
pZXL5	Thermosensitive plasmid. It carries a <i>mariner</i> transposon (Gm ^R) and a <i>mariner</i> transposase. Chlor ^R	JQ088279.1	Zhang <i>et al.</i> 2012

1014

1015

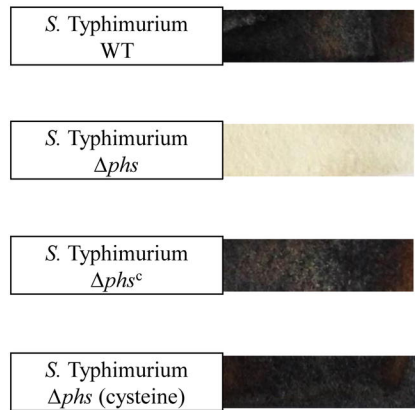
A**B**

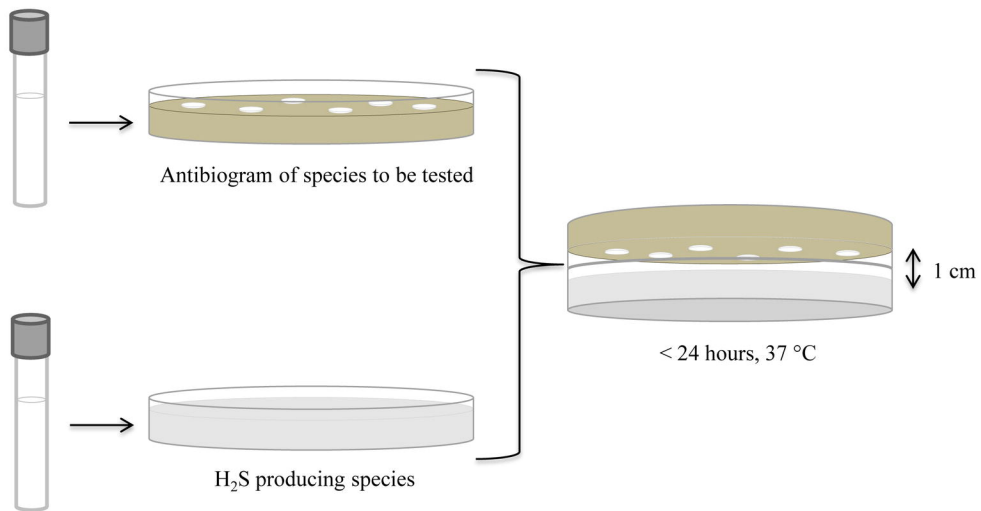
Salmonella Typhimurium
WT Δphs Δphs^c

+T-S
0 mM

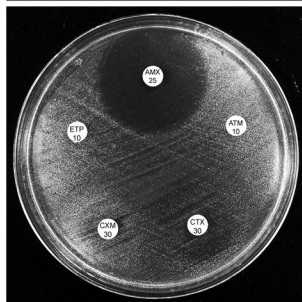
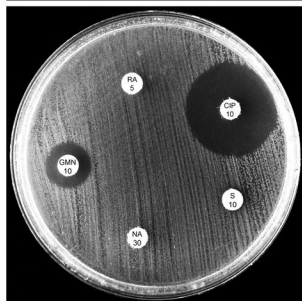
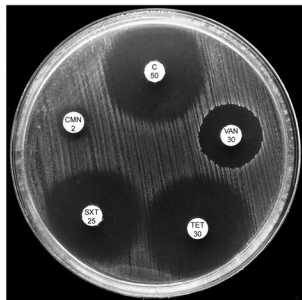


+T-S
20 mM

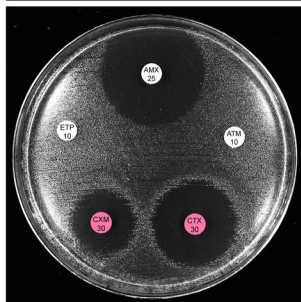
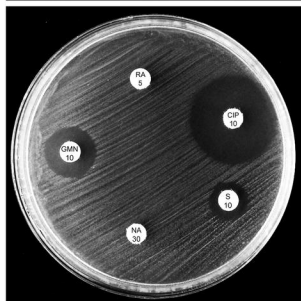
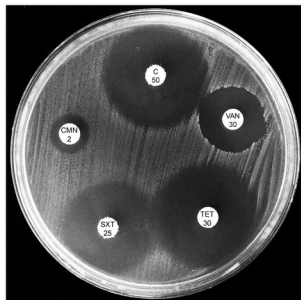
**C**



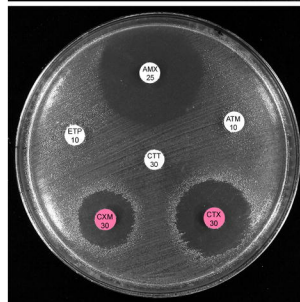
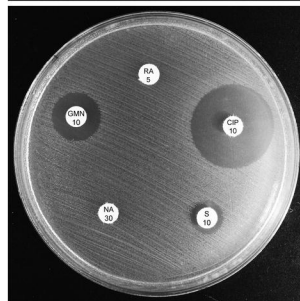
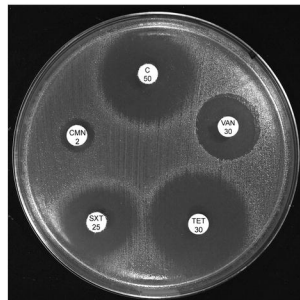
Control



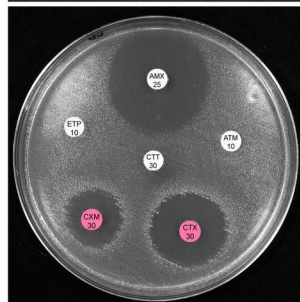
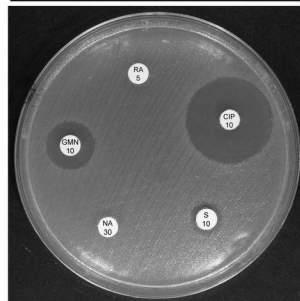
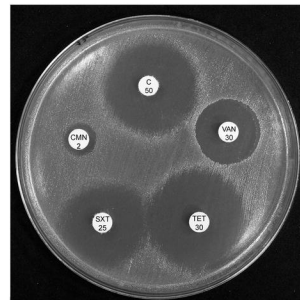
S. Typhimurium



E. coli (pH2S)



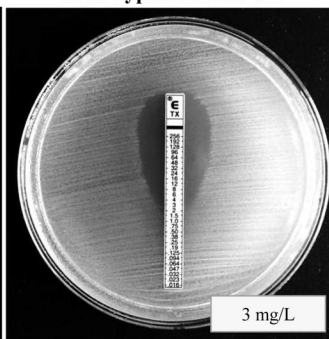
P. vulgaris



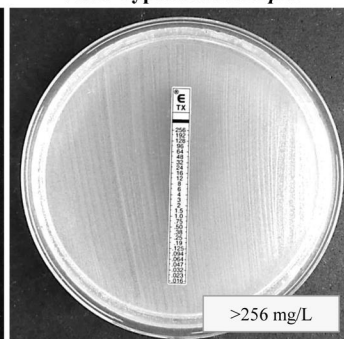
E. faecalis



E. faecalis
vs. *S. Typhimurium* WT



E. faecalis
vs. *S. Typhimurium* Δphs



Escherichia coli

WT WT (pH₂S)

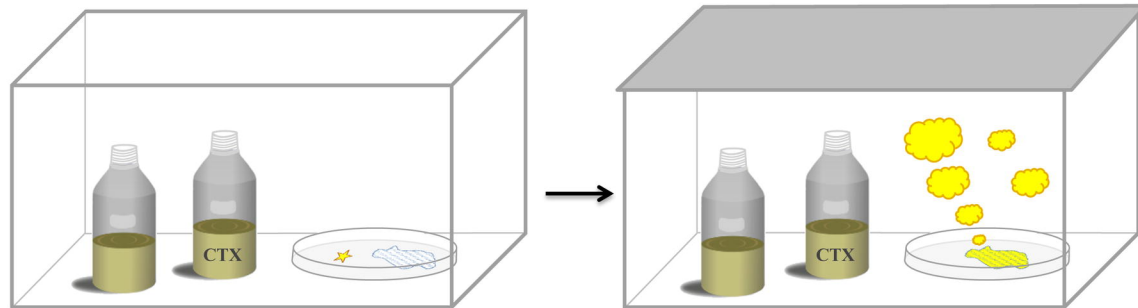


E. coli WT

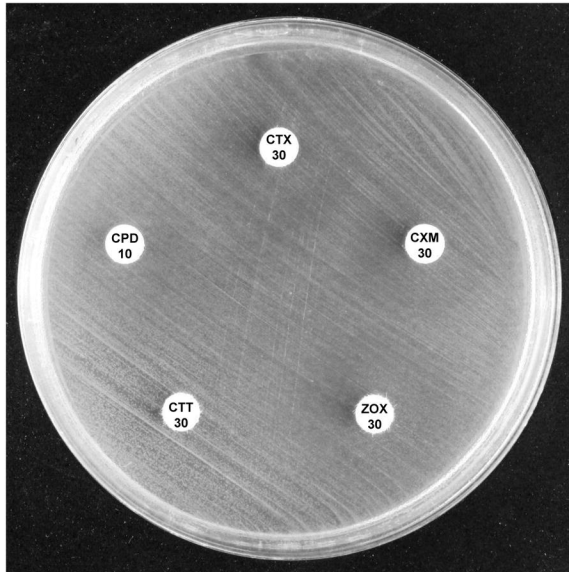


E. coli WT (pH₂S)

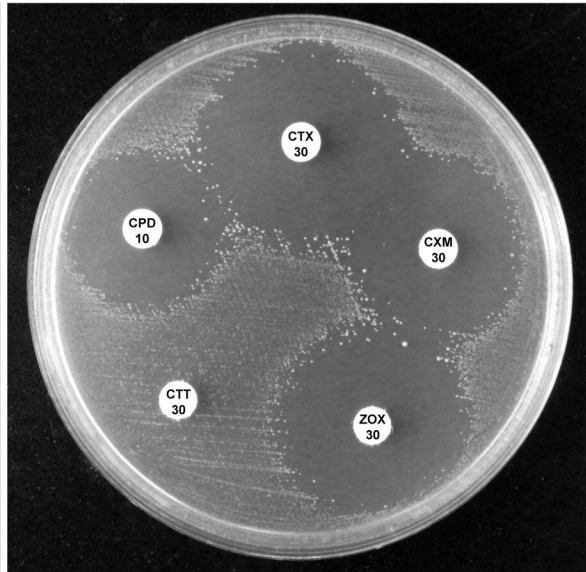




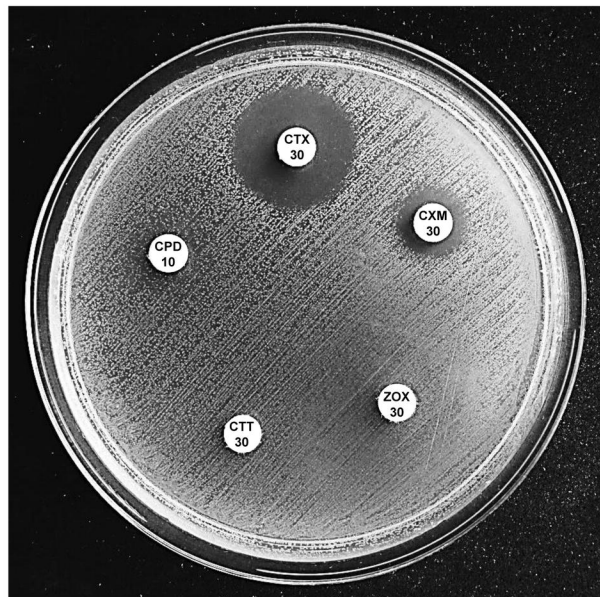
E. faecalis



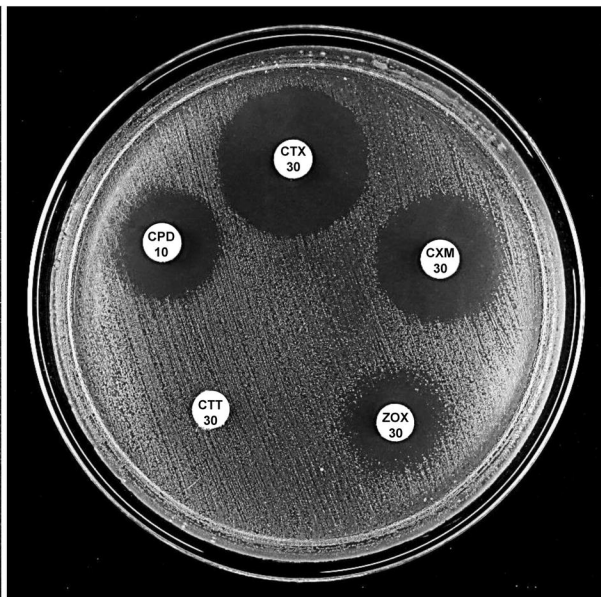
E. faecalis + H₂S

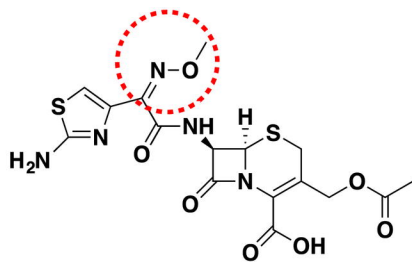


E. faecium

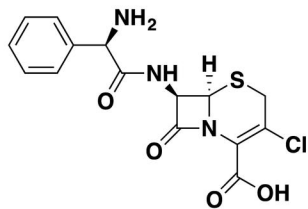


E. faecium + H₂S





Cefotaxime



Cefaclor

