Full title: H_2S mediates interbacterial communication through the air reverting intrinsic antibiotic resistance

Short title: H_2S producing bacteria can interfere with other species resistance to antibiotics

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Abstract

Hydrogen sulfide, a gas classically considered as a by-product of cellular metabolism, is today recognized as a crucial gasotransmitter in Eukaryotes. Moreover, most bacteria harbor the eukaryotic orthologous genes for H_2S synthesis, and these genes have been linked to different metabolic pathways.

Some bacteria, however, produce high amounts of H_2S in their extracellular space, a characteristic classically used for identification purposes. This is the case of *Salmonella* Typhimurium, which produces H_2S by its *phsABC* operon. Here we show that extracellular release of H_2S by *S.* Typhimurium is solely dependent on its *phsABC* operon. Furthermore, we show that *S.* Typhimurium and other H_2S-producing bacteria can interact with physically distant bacteria through H_2S production. We demonstrate how H_2S can revert intrinsic cephalosporin resistance of *Enterococccus faecalis* and *Enterococcus faecium* to complete susceptibility. This study constitutes a significant step in the study of bacterial interplay and niche competition. Furthermore, as H_2S releasing drugs have already been designed, our results open the way to future therapeutic alternatives for the treatment of infections caused by enterococci, multiresistant pathogens for which no treatments are clinically available.

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42 ## Author Summary

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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 (H_2S) 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 H_2S, 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.

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Introduction

It is well established that bacteria can communicate with each other through the diffusion of molecules in the media, what we know as quorum sensing [1]. On the other hand, in the 21st century it has been discovered that physically distant bacteria can also interact with each other or with distant organisms by releasing gaseous molecules [2], many of which were previously considered mere byproducts of bacterial metabolism. Ammonia [3], indole [4], trimethylamine [4] or acetic acid [5] are some these volatile compounds responsible for changes in motility, biofilm formation or antibiotic resistance. In addition, there are a few gases, called gasotransmitters, that are particularly interesting as they also play important roles in eukaryotic cells [6]. Gasotransmitters include mainly nitric oxide (NO), hydrogen sulfide (H_2S) and carbon monoxide (CO), and they have been connected to physiological and pathological conditions in cancer [7], the cardiovascular system [8,9], potassium channels [10], cellular ageing [11], animal hibernation [12] and grapes' senescence [13], among others.

In microbiology, NO and H_2S have received special attention as they enhance global antimicrobial resistance [14,15]. Bacteria have been shown to possess the orthologous genes to those found in eukaryotic cells for the production of these gases.

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

However, H_2S production in large amounts by specific pathways found in certain bacteria, *e. g.* from the *Salmonella*, *Citrobacter*, *Edwardsiella* and *Proteus* genera, has

been well known for more than 50 years [16]. Of the different H_2S synthesis mechanisms in bacteria, the most accurately characterized is the *phsABC* operon of *Salmonella* Typhimurium, which generates H_2S and sulfite through thiosulfate reduction. The purpose of this H_2S synthesis by *S.* Typhimurium is not well understood. It is well established that in the host's gut, thiosulfate can be oxidized to tetrathionate, which can be used by *S.* Typhimurium for respiratory purposes (reducing it again to thiosulfate) thanks to the *ttr* genes located in the pathogenicity island 2 (SPI-2) [17]. However, the benefits of a further reduction of thiosulfate to H_2S (instead of using it for further tetrathionate production) are very scarce [18].

Besides, if the function of this gas in bacteria is to enhance antibiotic resistance [15,19,20], it is intriguing that some species, like the gram positive *Enterococcus*, inhibit H_2S production by others [21]. However, this suggests that H_2S might be interacting with neighboring microorganisms, as it is the case with other molecules already mentioned.

Here we characterize the implication of the *phsABC* operon in H_2S synthesis by *S.* Typhimurium. As H_2S produced by the PHS pathway is released extracellularly, we studied if *S.* Typhimurium could communicate with other bacteria thanks to its H_2S production. We observed that it is not only the interaction that takes place but that *S.* Typhimurium can also revert other bacteria intrinsic antibiotic resistance, suggesting niche competition situations can be more complex than expected. Finally, we also demonstrate that H_2S abolishes antibiotic resistance. As H_2S releasing donors have already been developed, the combined application of antibiotics with H_2S requires future in depth studying.

Results

H_2S excretion relies entirely on *phsABC* action

Most bacteria possess H_2S producing genes, namely CBS/CSE and 3MST [15], together with cysteine desulfhydrases [22]. Nevertheless, a few bacterial species carry, in addition to the previously mentioned genes, an accessory and specific H_2S producing pathway. For example, *Salmonella* Typhimurium harbors the *phsABC* operon. To understand the role of this pathway, we deleted this operon (Fig 1A) and we measured the H_2S production of the deletion mutant *Δphs*, both in aerobic and anaerobic conditions.

Kligler media, which in the presence of H_2S forms a dark precipitate, turned completely black when *S.* Typhimurium wild-type strain was inoculated, whereas *Δphs* did not produce any black pigmentation of the media at all (Fig 1B).

Lead (II) acetate paper strips, which detect H_2S through the formation of black lead sulfide, allowed us to detect H_2S released not only to the media, but also outside of the media, as the strips were located above the culture medium. Again, we observed that the strips turned completely black with the WT culture, while incubation of *Δphs* strain barely caused any staining (Fig 1C).

In both cases, complementation of the strain with the pSB74 plasmid, which will be called pH_2S as it bears the entire *phsABC* operon [23], restored H_2S production at WT levels in the new strain *S.* Typhimurium Δphs^c (Fig 1).

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

H_2S has been linked to antibiotic resistance, via the H_2S synthesis pathways found in most bacteria [15,20]. However, this has not been demonstrated for the *S.* Typhimurium specific PHS pathway, even though this is considered the main source of H_2S in this species [23,24]. Therefore, we carried out various antimicrobial susceptibility tests (S1 Fig, **Error! Reference source not found.**S1 and S2 Tables), but no differences were observed in any case. Growth curves showed no difference either in growth rate between WT and *Δphs* strain (S1 Fig).

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

H_2S produced by means of the PHS pathway by *S.* Typhimurium is largely released from the cell and does not appear to be implicated its own growth or antibiotic resistance. Thus, we hypothesized that H_2S may have an external role on the antibiotic resistance pattern of neighboring bacteria when *S.* Typhimurium interacted with them.

We designed a model to effectively assess signaling between physically distant bacteria (Fig 2). This method consists in preparing antibiograms of the species to be tested and confronting them against another Petri dish in which the H_2S producing strain has been plated with a cotton swab. Subsequently, plates were incubated for a maximum of 24 hours.

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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 H_2S 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 H_2S.

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 H_2S 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 H_2S produced by the *phsABC*
181 operon that *S.* Typhimurium induces *Enterococcus* killing. *S.* Typhimurium (pTrc99a),

i.e., the strain carrying the empty vector pTrc99a, was used as a negative control to ensure that this plasmid had no influence on the effect. As we established that *phsABC* is a predominant mechanism for extracellular H_2S release from this species, we conclude that this operon is crucial for the remote induced killing of neighbor bacteria by *S.* Typhimurium.

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

Strains	+ T-S 2 mM			+ T-S 20 mM			+ Cys 2 mM			+ T-S 20 mM Anaerobiosis		
	ZOX	CTX	CEC									
E. faecalis	6	6	16	-			-			-		
***vs. S.* Typhimurium WT**	28	30	19	25	33	16	27	30	17	22	26	19
vs. S.* Typhimurium Δ*phs	6	6	14	6	6	12	27	30	17	6	6	14
***vs.* Δ*phs* (pTrc99a)**	6	6	14	6	6	13	23	23	16	ND		
***vs.* Δ*phs* (pH_2S)**	25	28	16	32	34	16	25	27	17	ND		

ND: not determined

E. faecalis JH2-2 is plated on Tryptone-Soya Agar (TSA). *S.* Typhimurium strains are plated on TSA supplemented with thiosulfate 2 or 20 mM. Halos are expressed in mm. Inhibition zones displayed only in the presence of H_2S are underlined. Ceftizoxime (ZOX), Cefotaxime (CTX), Cefaclor (CEC).

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

To identify if the product of the *phsABC* operon was having an effect in adjuvancy with other factors from *S.* Typhimurium, we transformed *E. coli* MG1655 strain with the pH_2S plasmid. We show that the *phsABC* operon is sufficient for the increased production of H_2S by *E. coli* MG1655 (Fig 5), implying that no further networks or genes present in *Salmonella* are necessary. Lead (II) acetate paper strips were stained in a similar way in *E. coli* (pH_2S) and *S.* Typhimurium. When *E. coli* (pH_2S) was incubated in Kligler media, we observed a lesser amount of staining than in *S.* Typhimurium. This can be explained by the fact that *S.* Typhimurium also harbors the anaerobic sulfite reductase (*asr*) that generates further H_2S from the reduction of the sulfite previously generated by PHS.

Furthermore, when we confronted different *E. coli* strains with *E. faecalis*, we observed that only *E. coli* (pH_2S) could induce the susceptibility of *E. faecalis* to cephalosporins (Fig 3), showing that the *phsABC* function is sufficient for the resistance reversion phenomenon carried out by H_2S and that no secondary metabolites produced by *S.* Typhimurium are necessary.

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

H_2S is responsible for the reversion of the intrinsic resistance to cephalosporins in *E. faecalis*

The deletion of the *phsABC* operon of *S.* Typhimurium could have further consequences on the bacteria (*e. g.*, affecting other metabolic pathways) that could ultimately be responsible for the distant effect observed on *Enterococcus* rather than the H_2S itself. To rule out this hypothesis, we tested other sources of H_2S. First, we carried out confrontation experiments in the presence of cysteine, the substrate of the 3MST pathway [15], present in many bacteria including *S.* Typhimurium. Even if we have proved that under basal conditions PHS is the predominant mechanism for extracellular production of H_2S, we hypothesized that addition of external cysteine should increase the production of H_2S by this pathway, as high levels of cysteine are toxic for the cell [22]. Upon adding cysteine, we observed that H_2S is again detected extracellularly in the *Δphs* mutant (Fig 1) and that this mutant is able to revert *Enterococcus* cephalosporin resistance in a similar extent as the wild-type *S.* Typhimurium (Table 1). We confronted *E. faecalis* with *Proteus vulgaris*, another species commonly known for its extracellular production of H_2S [22] and *P. vulgaris* proved to be equally efficient in inducing cephalosporin susceptibility in *E. faecalis* (Fig 3).

Finally, we checked if pure H_2S from a chemical origin also acts synergistically with cephalosporins. NaHS is a widely used H_2S source [15,25]. We designed a technique in which we placed NaHS physically separated from the enterococci. We put our antibiograms in a glass chamber, next to an empty Petri dish in which we placed 0.1 grams of NaHS and 2 mL of ultrapure water. Once we sealed the chamber, we gently shook it to put the crystal into contact with the water so that the gaseous H_2S could be released into the chamber (Fig 6).

With this experiment, we proved that the resistance of *E. faecalis* to cephalosporins could be reversed by pure H_2S. In addition, we also showed that other *E. faecalis* strains are affected in the same way. Most notably, V583, the first vancomycin resistant clinical isolate, displayed the same phenotype (Fig 7).

E. faecium cephalosporin resistance is also reverted in the presence of H_2S

Next, we were interested in testing the effects of H_2S on the other pathogenic enterococcus , *E. faecium*, as in the last decade this species has acquired even higher clinical relevance than *E. faecalis*, partially because most of the enterococcal vancomycin resistant clones currently detected are *E. faecium* [26]. We have verified that this species is also susceptible to the synergic effect of H_2S with cephalosporins (Fig 8), further suggesting the potential application of this combination in clinical settings.

Reversion of the intrinsic resistance of enterococci in the presence of H_2S takes place specifically with methoxy-imino cephalosporins

As we can notice in Figs 3, 7 and 8, and Table 1, synergy between H_2S and cephalosporins does not take place with every compound of this family of antibiotics. By performing antibiograms of a set of 20 cephalosporins, including 1^{st} to 4^{th} generation cephalosporins, we have observed that in the presence of H_2S *E. faecalis* demonstrates a significant susceptibility against cefotaxime, ceftriaxone, cefuroxime, cefpodoxime and ceftizoxime (Table 2). These drugs, frequently used in clinical settings, contain a common methoxy-imino group in their structure (Fig 9), which is not present in other

cephalosporins within the family, suggesting that this motif might be a key in the synergy displayed between H_2S and cephalosporins.

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

	TSA	H_2S 0.1 g
Cefaclor 30	10	13
Cefadroxil 30	13	13
Cephalexin 30	8	8
Cefalotin 30	16	16
Cefamandole 30	14	15
Cefazolin 30	14	16
Cefepime 30	13	15
Cefotaxime 30	6	21
Cefotetan 30	6	6
Cefoxitin 30	6	6
Cefpirome 30	22	25
Cefpodoxime 10	6	20
Cefprozil 30	21	23
Cefradine 30	12	13
Cefsulodin 30	6	6
Ceftizoxime 30	6	16
Ceftriaxone 30	6	16
Cefuroxime 30	6	20

Methoxy-imino cephalosporins are underlined.

H_2S and cephalosporins kill enterococci

Finally, to further characterize the H_2S-cephalosporins synergy, *E. faecalis* JH2-2 was grown in liquid media in the presence and absence of H_2S and/or cefotaxime (CTX).

By performing a Two-way ANOVA (α = 0.05; P <0.001; F = 68,46; df = 17, 28) and a post-hoc Tukey–Kramer analysis, used for single-step multiple comparison of all pair of means, we observed that there is a strong synergistic effect between H_2S and CTX (P <

290 0.001), not only by inhibiting bacterial growth in the presence of both compounds, but
291 also by producing a bactericidal effect (Fig 10, S4 Table). With this analysis we
292 confirmed that, in the concentration range used in our study, H_2S does not affect
293 bacterial growth on its own. Even if enterococci were resistant to cephalosporins, a
294 minor effect of cefotaxime on bacterial growth was noticeable, but this is something
295 that could be observed with many antibiotic discs in antibiograms or other antibiotic
296 susceptibility tests.

297 Preliminary experiments with cefaclor, a non-methoxy-imino cephalosporin, or with
298 nalidixic acid, a quinolone against which *E. faecalis* is resistant, showed no synergy
299 with H_2S (data not shown), indicating that the displayed phenotype is specific of certain
300 antibiotics.

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302 **The persulfidome analysis of *E. faecalis* reveals potential targets for H_2S**

303 We carried out random transposition mutagenesis of *Enterococcus* under cephalosporin
304 selective pressure, constructing a high-density transposon mutant library. However, no
305 mutants were obtained using this approach. This suggests that a single gene alteration is
306 not enough to prevent resistance reversion, or that this alteration has to take place in an
307 essential gene. Therefore we wanted to address posttranslational changes of the protein
308 that are induced by H_2S.

309 Signaling by H_2S is now widely linked to an oxidative posttranslational modification of
310 cysteine residues (RSH) called persulfidation (RSSH) (alternatively S-sulfhydration)
311 [27–29]. Exposure of cells to H_2S results in an increase of protein persulfidation
312 [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 H_2S.

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 H_2S. 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 H_2S.

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333 **Table 3. Persulfidome of *E. faecalis*.** List of proteins of *E. faecalis* JH2-2 detected to interact
334 with H_2S.

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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

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	
Q837E3	EF0900	Aldehyde-alcohol dehydrogenase	340
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	

Discussion

In this work we present significant progress in the study of H_2S as a gasotransmitter and its link to antibiotic resistance.

S. Typhimurium is one of the classic H_2S producing bacterial species, via its *phsABC* operon [18]. It is known that its capacity to reduce sulfur compounds provides *Salmonella* with a growth advantage when colonizing the digestive tract [17,33]. However, the physiology and involvement of the *phsABC* operon in antibiotic resistance, which has both a significant scientific and clinical interest, is not described.

The aim of this study was to understand the role and co-regulation of the H_2S-producing pathways in *S.* Typhimurium, its possible role in microbial ecology and its effects on other bacteria. By knocking-out the *phsABC* operon, we have found that the PHS pathway is the predominant mechanism for H_2S production in this species. Other pathways may or may not be active, but the lack of the PHS pathway is not compensated by other mechanisms.

We have demonstrated that the *phsABC* operon is not involved in own antibiotic resistance, even though it is the main source of H_2S in *S.* Typhimurium. This suggests that the modifications in antibiotic resistance previously described in this species [20,34] are not due to the H_2S itself. In fact, these authors state that the accumulation of H_2S in their experiments is due to either a decrease in cysteine synthesis or an increase in its catabolism. Cysteine is a well-known inducer of the Fenton reaction and the H_2S-mediated augmentation of antibiotic resistance is associated with oxidative stress protection [15]. This means that H_2S itself may be irrelevant for antibiotic resistance in *S.* Typhimurium, which explains the absence of differences between WT and *Δphs* strains regarding every aspect we have tested (except for the production of H_2S itself).

As we could not detect any intracellular role for H_2S in *S.* Typhimurium, but we had verified that the *phsABC* operon was used to generate large amounts of H_2S, we hypothesized that this H_2S may have an extracellular effect, that is, on neighboring bacteria. Moreover, it is known that thiosulfate is abundant in the digestive tract [17], suggesting that the *phsABC* operon should be active.

When *E. faecalis* was tested, instead of observing an enhancement of antibiotic resistance by H_2S, as previously described [15,20], we observed that this gas is capable of disrupting the intrinsic antibiotic resistance of *Enterococcus*. The MIC of cefotaxime in *E. faecalis* was dramatically decreased and resistance to other cephalosporins was similarly reverted, both in *E. faecalis* and *E. faecium* multiresistant enterococci, responsible for endocarditis and septicemia in hospitals worldwide, are ranked by the WHO among the pathogens for which effective therapies are critically needed [35]. In addition, the use of certain antibiotics is a predisposing factor for suffering an overgrowth and subsequent infections by *Enterococcus* [36,37]. Therefore, as pharmacological donors of H_2S have already been developed [38–41], our work gives new hope for the treatment of enterococcal infections. H_2S donor drugs could be administered together with cephalosporins to effectively kill multidrug resistant enterococci in patients. In addition, unraveling the mechanism of action of H_2S inside *Enterococcus* could lead to the design of molecules that emulate H_2S function.

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

In our experiments, we observed that H_2S exhibits bactericidal synergy with a select group of cephalosporins containing a methoxy-imino group in its structure. This residue improves the drug's stability to β-lactamases and improves the ability of the drug to cross the external membrane of gram-negative bacteria [42]. Clearly, these properties do not account for its efficacy in the presence of H_2S, as β-lactamase production by *Enterococcus* is anecdotic [43]. But, interestingly, many of the studies regarding cephalosporin resistance in enterococci were obtained with this specific group of methoxy-imino cephalosporins [44–49]. Thus, H_2S might be affecting some of the pathways already described for *Enterococcus* cephalosporin resistance.

These pathways include a penicillin-binding protein that presents low affinity to these drugs [50], the MurAA enzyme involved in peptidoglycan synthesis [47], mutations of the β-subunit of the RNA polymerase [49], CroR/CroS and IreK/IreP two-component systems [45,48], or alterations in the thymidylate synthesis pathway [44], among others. Still, most of these studies do not unravel the final mechanisms governing cephalosporin resistance and they suggest a common currently undescribed mechanism [47,48,51]. Besides, in our experiments we have also observed an induction of aminoglycosides susceptibility by H_2S (S3 Table), which it has not been observed by other authors. Therefore, H_2S could be acting through a different pathway or upstream of a common mechanism, a theory that would be also supported by the fact that no known protein involved in cephalosporin resistance has been detected in the persulfidome (Table 3). In any case, aminoglycosides constitute another group of last resort antibiotics widely used in clinical settings and for which enterococci present a modest level of intrinsic resistance [52]. It would be interesting to study in depth this additional synergy between H_2S 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 H_2S 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 H_2S, *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 H_2S 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 H_2S 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 H_2S production. We show how
431 *Salmonella* and *Proteus*, through their H_2S 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 H_2S
434 aerial communication, showing how a bacterium can exert an effect with lethal
435 consequences on another bacterium through H_2S, a gasotransmitter with vital functions
436 and abundant in the human body and to date considered a beneficial gas for bacteria.

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Competing interests

The authors claim no conflict of interest.

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Authorship

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

Competing interests

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

Materials and Methods

Strains, media and reagents

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

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

Table 4. Bacteria used in this study.

Strain	Abbreviated name	Description	Reference
Enterococcus			
E. faecalis VE14089	V583 WT	V583 strain cured from its plasmids	Rigottier-Gois *et al.* 2011
E. faecalis JH2-2	JH2-2 WT	Laboratory strain	Jacob & Hobbs 1974
E. faecium ATCC19434	*E. faecium*	Type strain	ATCC
***Salmonella* Typhimurium**			
S. enterica enterica serovar Typhimurium ATCC14028 wild type	*S.* Typhimurium WT	Type strain	ATCC
S. Typhimurium *ΔphsABC kan*^{R}	*kan*^{R}	*phsABC* operon substituted with the kanamycin resistance cassette	This work
S. Typhimurium *ΔphsABC*	*Δphs*	*phsABC* operon deletion mutant	This work
S. Typhimurium *ΔphsABC* (pTrc99a)	*Δphs* (pTrc99a)	*Δphs* mutant transformed with pTrc99a	This work
S. Typhimurium *ΔphsABC* (pH_sS)	*Δphs*^{c}	*Δphs* mutant transformed with pSB74	This work
Escherichia coli			
E. coli MG1655	*E. coli* WT	Laboratory strain	ATCC
E. coli MG1655 (pH_sS)	*E. coli* (pH_2S)	MG1655 strain transformed with pSB74	This work

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

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

Primers used and DNA manipulation

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

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

Plasmids used in this study

Plasmid pTrc99a was kindly donated by Professor Javier Turnay from the Biochemistry Department of the Complutense University of Madrid. pSB74 [23] was acquired from Addgene (plasmid #19591). Plasmid pKD13 (GenBank Accession number AY048744) was used as a PCR template in order to construct the deletion mutants and it contains an FLP recombination target (FRT)-flanked kanamycin resistance (*kan*) gene. Arabinose-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 Δ*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 H_2S 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 ºC.

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

turbidity suspension of the H_2S producing species (or its mutant) was spread evenly over the entire surface of a TSA plate, supplemented with sodium thiosulfate T-S 2 mM, T-S 20 mM or Cys 2 mM prepared within the last seven days. Then, antibiograms of the strains to be tested were prepared in TSA, attending to official guidelines. Finally, lids were removed and both plates were fixed together with two pieces of adhesive tape, facing each other. The edges zone was "sealed" with air permeable Parafilm M (Bemis Company Inc, Oshkosh, WI). Plates were incubated at 37 ºC for 24 hours maximum. Anaerobiosis conditions were achieved by using the GENBag system from bioMérieux.

Growth curves

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

Experiments incubated with H_2S

For experiments in the presence of H_2S (Fig 6), *E. faecalis* antibiograms were prepared in TSA plates following official guidleline and were placed in a glass chamber of 2 liters volume. Next to them, we placed the base of an empty Petri dish in which we positioned 0.1 grams of NaHS and, separated, 2 mL of ultra-pure water. Then, we

566 hermetically sealed the chamber and, only afterwards, NaHS and water were put in
567 contact by gently shaking the chamber. Antibiograms were incubated for a maximum of
568 24 hours at 37 ºC.

569

570 **Time killing experiments**

571 10 µL from an o.n. culture of *E. faecalis* in TSB were inoculated to 15 mL glass bottles
572 containing 10 mL of TSB. When required, antibiotics at their proper concentration were
573 added. Bottles were incubated at 37 ºC and 100 rpm. Those bottles that required H_2S
574 presence were placed in a glass hermetic chamber the same way as with experiments
575 from the previous section (Fig 6). At 12 hours proper dilutions were plated for colony
576 forming units (CFU) counting. From that moment on, all bottles were incubated under
577 normal conditions until 24 hours, when dilutions were plated again.

578

579 **Random transposition mutagenesis**

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

586 Bacteria containing pZXL5 were incubated o.n. at 28 ºC in BHI media containing
587 gentamicin 300 mg/L and chloramphenicol 10 mg/L. Afterwards, a 1:2000 dilution was
588 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 ºC 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 ºC and 150 rpm o.n., obtaining a library of mutants that
595 was stored at -80 ºC for future experiments.

596 By incubating in the presence of gentamicin, cefotaxime and H_2S, we attempted to
597 select only those mutants that resisted the combined action of H_2S 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 ºC and 100 rpm. Then, each sample was split in two and, to one of each set,
605 H_2S 250 µM (freshly prepared as previously described) was added. The four bottles
606 were incubated for 30 minutes at 37 ºC 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 ºC.

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

Proteins were precipitated with $CHCl_3$/MeOH/H_2O precipitation (1/4/4, v/v), washed twice and resuspended in HEPES buffer pH 7.4 containing SDS up to 0.5%. Samples were adjusted to equal protein concentration. Biotinylated proteins were bound to streptavidin agarose beads, and persulfidated targets eluted by DTT 0.5 mM (Fig 11).

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

nanoLC/MS

Protein samples were run on SDS-PAGE (10 %) but protein separation was stopped once proteins have entered the resolving gel. After colloidal blue staining, each lane was was cut into 1 mm x 1 mm gel pieces. The gel pieces were destained in 25 mM ammonium bicarbonate 50% ACN, rinsed twice in ultrapure water and shrunk in ACN for 10 min. After ACN removal, gel pieces were dried at room temperature, carbamidomethylated and covered with the trypsin solution (10 ng/µL in 50 mM NH_4HCO_3), rehydrated at 4°C for 10 min, and finally incubated overnight at 37°C. The gel pieces were then incubated for 15 min in 50 mM NH4HCO3 at room temperature with rotary shaking. The supernatant was collected, and an H_2O/ACN/HCOOH (47.5:47.5:5) extraction solution was added onto the gel pieces for 15 min. The extraction step was repeated twice. The pooled supernatants were dried in a vacuum

centrifuge and then resuspended in 40 µL 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-µm-inner diameter x 5-mm C18 PepMapTM trap column (LC Packings) at a flow rate of 30 µL/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 x 106 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 x 105 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) H_2S 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 H_2S
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* (pH_2S), *Proteus vulgaris* CECT174. H_2S
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 H_2S
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. H_2S production by *E. coli* WT and *E. coli* p(H_2S).**

702 H_2S production was measured with Kligler media (left) and lead acetate strips (right).

703

704 **Fig 6. Method for incubation with chemical H_2S.**

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 H_2S to the atmosphere upon contact with
708 H_2O (B). CTX: cefotaxime.

709

710 **Fig 7. Antibiograms of *E. faecalis* in the presence of H_2S.**

711 *E. faecalis* V583 was incubated in a chamber in the presence and abscence 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. Antibiograms of *E. faecium* in the presence of H_2S.**

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

Fig 9. Chemical structure of cephalosporins.

In cefotaxime the methoxy-imino residue is emphasized.

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

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

Fig 11. Identification of he complete persulfidome of *E. faecelis*

(A) Workflow used for the specific labeling of persulfide residues. Biotin maleimide binds only to thiol and persulfide motifs. Bound proteins are separated from others by streptavidin magnetic beads. DTT treatment specifically cleaves disulfide bridges with biotin maleimide, rendering the original S-sulfhydrated proteins available for subsequent procedures. (B) SDS-PAGE of *E. faecalis* total protein extraction incubated in the presence and absence of H_2S and CTX.

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

The use of cephalosporins or other antibiotics to which *E. faecalis* and *E. faecium* are resistant promotes these species overgrowth in the host's digestive tract, facilitating their dissemination and the development of enterococcal infections (upper panel). Nevertheless, a cephalosporin-resistant *S.* Typhimurium strain in the niche could induce the killing of enterococci by H_2S production, displacing the latter and colonizing the gut as a previous step for infection (bottom panel).

745

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Supplementary material

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

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

S1 Table. Antibiograms of *Salmonella* Typhimurium WT and Δ*phs* strain, in Tryptone Soy Agar (TSA), in the presence and absence of thiosulfate 20 mM (T-S).

	***S.* Typhimurium WT**		***S.* Typhimurium *Δphs***	
	TSA	TSA + T-S 20	TSA	TSA + T-S 20
Amox. + Clavulanic acid 30	31	28	32	29
Cefaclor 30	31	30	29	31
Cefotaxime 30	34	34	34	34
Cefpodoxime 10	28	29	30	27
Cefoxitin 30	26	25	29	25
Imipenem 10	33	32	33	32
Aztreonam 30	38	34	34	34
Apramycin 15	13	10	13	9
Gentamicin 10	16	14	17	14
Neomycin 30	16	14	15	13
Netilmicin 30	19	17	19	17
Estreptomycin 10	12	7	12	7
Ciprofloxacin 10	35	37	35	36
Sulpha - Trimetoprim 25	29	30	29	30
Tetracycline 30	27	27	26	27
Clindamycin 2	6	6	6	6
Colistin 10	14	14	13	14
Cloramphenicol 50	25	27	28	28
Erythromycin 15	7	6	6	7
Mupirocin 5	6	6	6	6
Rifampin 5	9	7	9	9
Vancomycin 30	6	6	6	6
Fosfomycin 200	36	30	33	31

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

MIC (mg/L)	*S.* Typhimurium WT	*S.* Typhimurium *Δphs*
Ampicillin	1	1
Cefotaxime	0.125	0.062
Streptomycin	128	64
Cloramphenicol	8	8
Ciprofloxacin	0.031	0.031

975

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

	TSA	TSA +H_2S 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

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

TUKEY-KRAMER POST-HOC ANALYSIS

		WT 0	WT 12	WT 24	H_2S 0	H_2S 12	H_2S 24	CTX 0	CTX 12	CTX 24	H_2S-CTX 0	H_2S-CTX 12	H_2S-CTX 24
WT	0		**<.0001**	**<.0001**	1.0000	<.0001	<.0001	1.0000	<.0001	<.0001	1.0000	0.0081	0.0004
	12			1.0000	<.0001	0.9257	0.9560	<.0001	**0.0188**	0.0137	<.0001	**<.0001**	<.0001
	24				<.0001	0.9984	0.9996	<.0001	0.0670	0.0629	<.0001	<.0001	**<.0001**
H_2S	0					**<.0001**	**<.0001**	1.0000	<.0001	<.0001	1.0000	0.0124	0.0006
	12						1.0000	<.0001	0.3500	0.3149	<.0001	**<.0001**	<.0001
	24							<.0001	0.2910	0.2520	<.0001	<.0001	**<.0001**
CTX	0								**<.0001**	**<.0001**	1.0000	0.0206	0.0010
	12									1.0000	<.0001	**<.0001**	<.0001
	24										<.0001	<.0001	**<.0001**
H_2S - CTX	0											**0.0142**	**0.0007**
	12												0.9914
	24												

S5 Table. Primers used in this study.

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

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

Plasmid name	Description	GenBank Access number	Reference
In-frame genes deletion			
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
H_2S production			
pTrc99a	Empty vector		Bang *et al.* 2000
pSB74 (pH_2S)	pTrc99a + *phsABC*	-	Bang *et al.* 2000
Random transposition mutagenesis			
pZXL5	Thermosensitive plasmid. It carries a *mariner* transposon (Gm^{R}) and a *mariner* transposase. $Chlor^{R}$	JQ088279.1	Zhang *et al.* 2012

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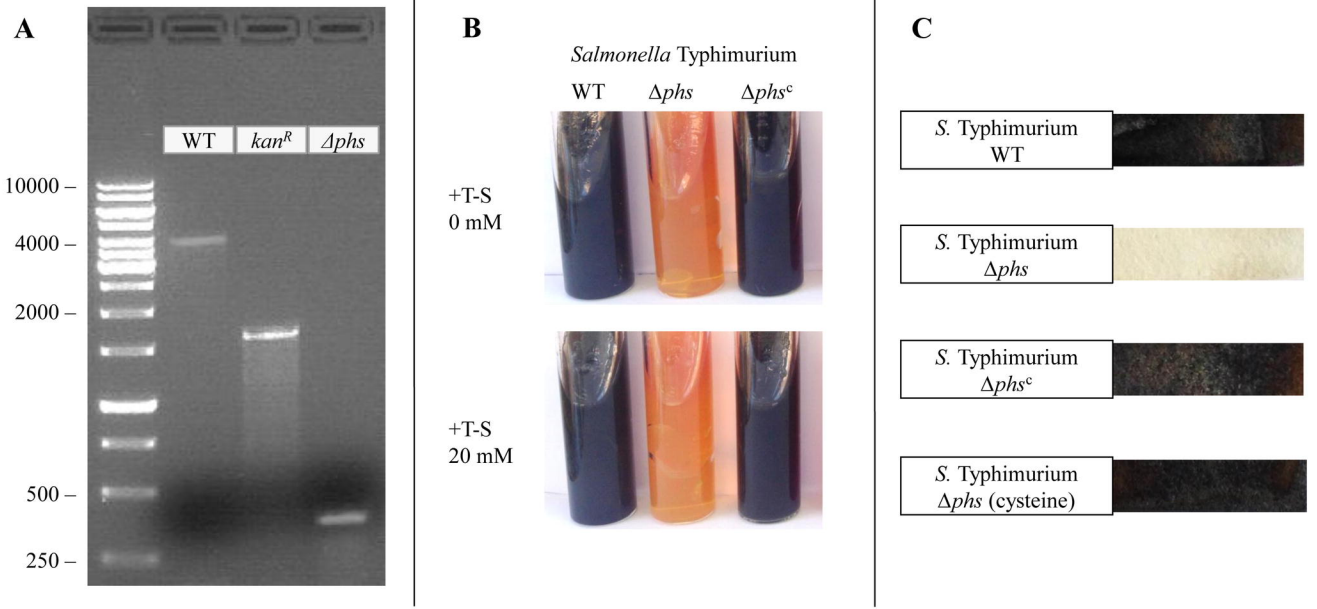
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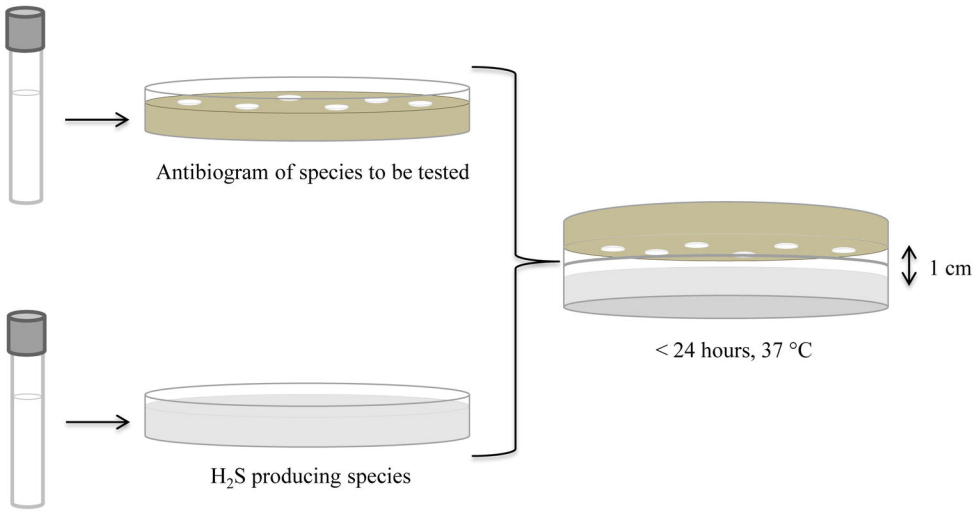
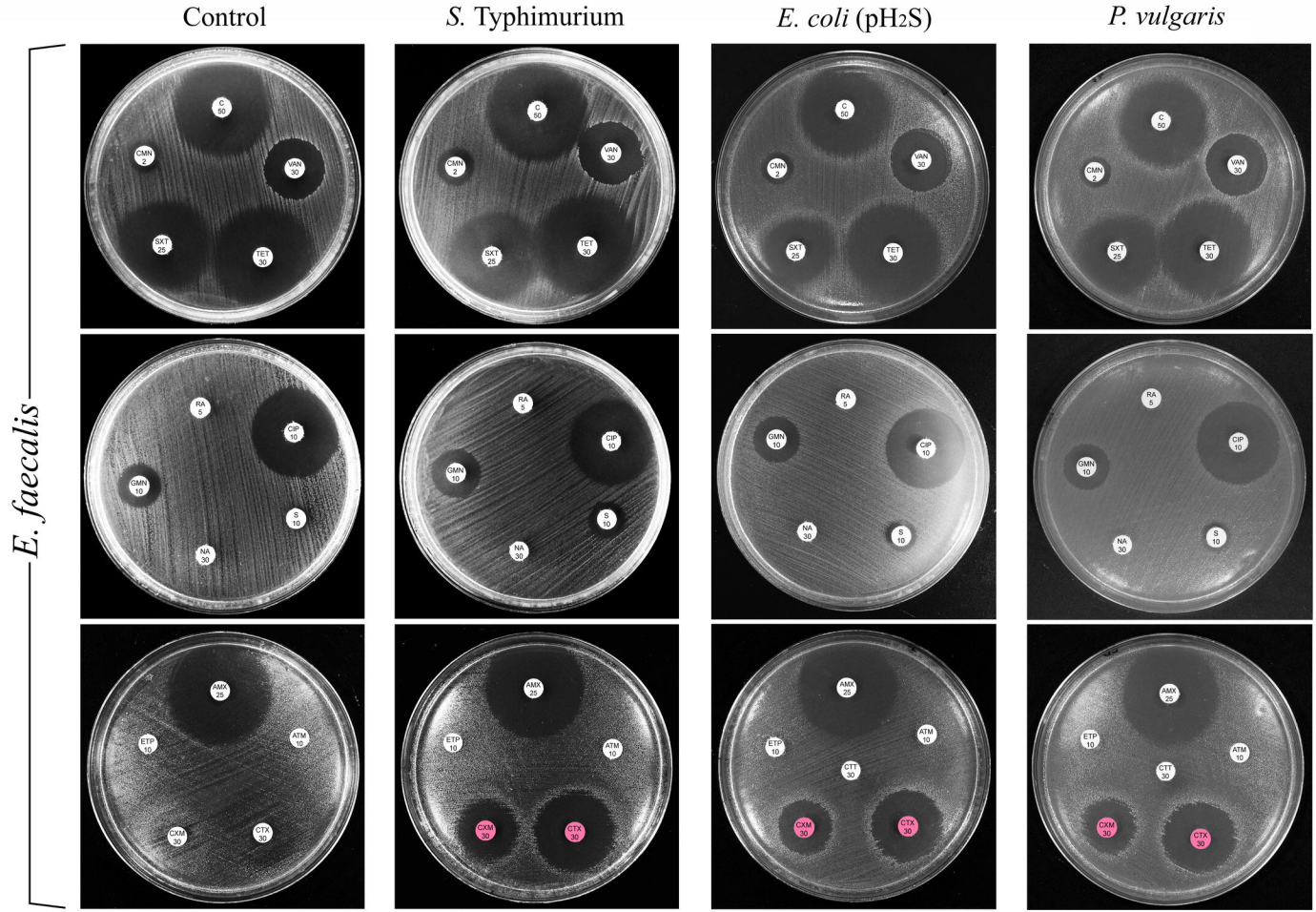
WT
kanR
Δphs
10000 –
4000 –
2000 –
500 –
250 –
B
Salmonella Typhimurium
WT
Δphs
Δphsc
+T-S
0 mM
+T-S
20 mM
C
S. Typhimurium
WT
S. Typhimurium
Δphs
S. Typhimurium
Δphsc
S. Typhimurium
Δphs (cysteine)


Antibiogram of species to be tested
H_2S producing species
1 cm
< 24 hours, 37 °C


Control
S. Typhimurium
E. coli (pH2S)
P. vulgaris
E. faecalis
C 50
CMN 2
VAN 30
SXT 25
TET 30
RA 5
CIP 10
GMN 10
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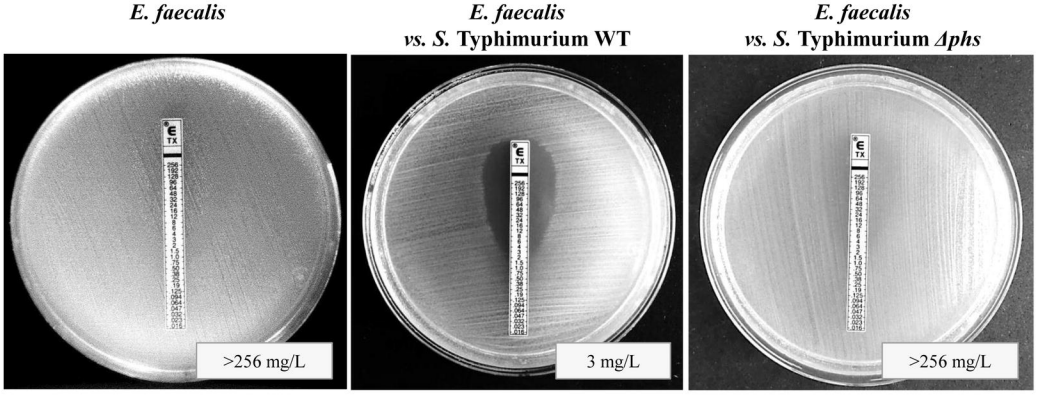
NA 30
AMX 25
ETP 10
ATM 10
CXM 30
CTX 30
CTT 30

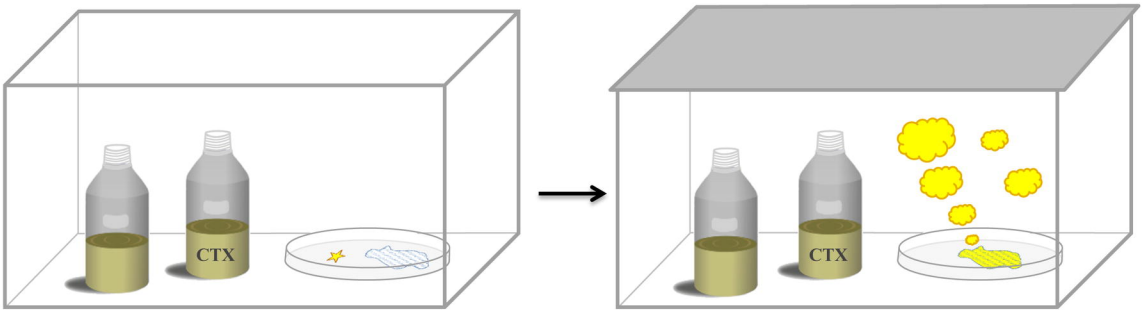

E. faecalis
E. faecalis
vs. S. Typhimurium WT
E. faecalis
vs. S. Typhimurium *Δphs*
>256 mg/L
3 mg/L
>256 mg/L

Escherichia coli

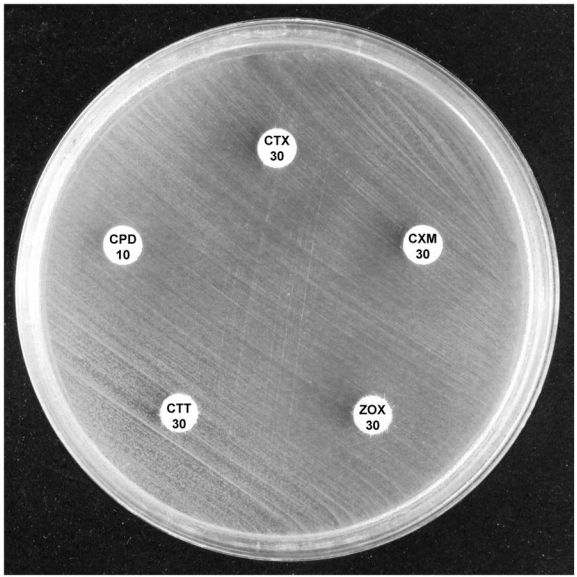
WT WT (pH_2S)

E. coli WT

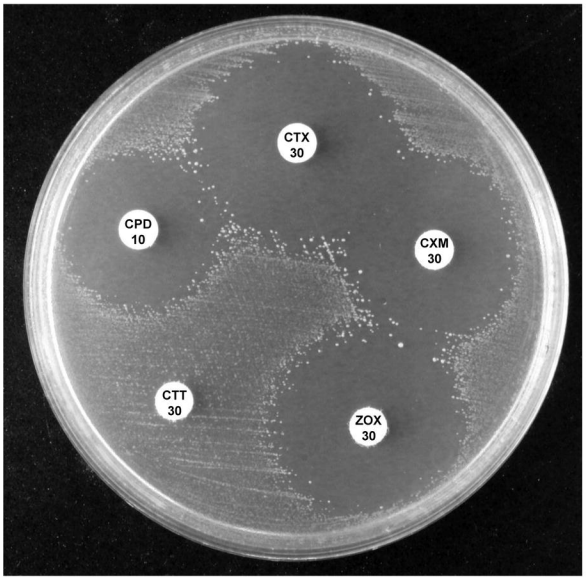
E. coli WT (pH_2S)


CTX
CTX

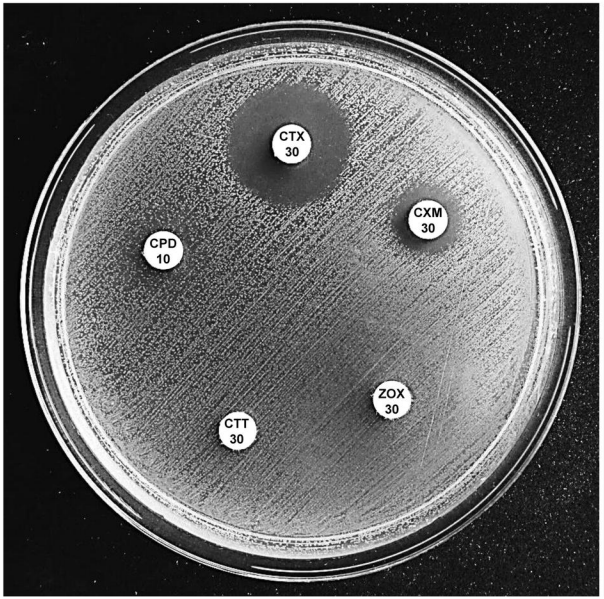
E. faecalis



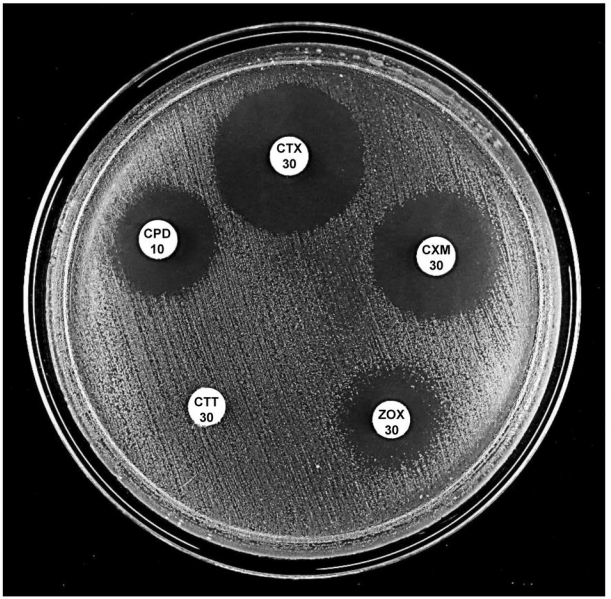
E. faecalis + H_2S

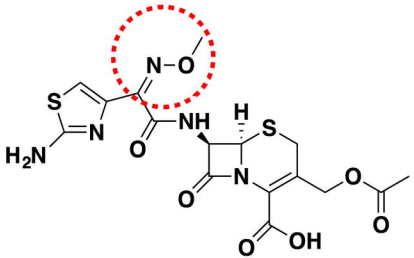


E. faecium



E. faecium + H_2S





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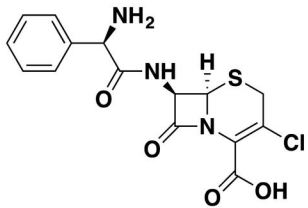

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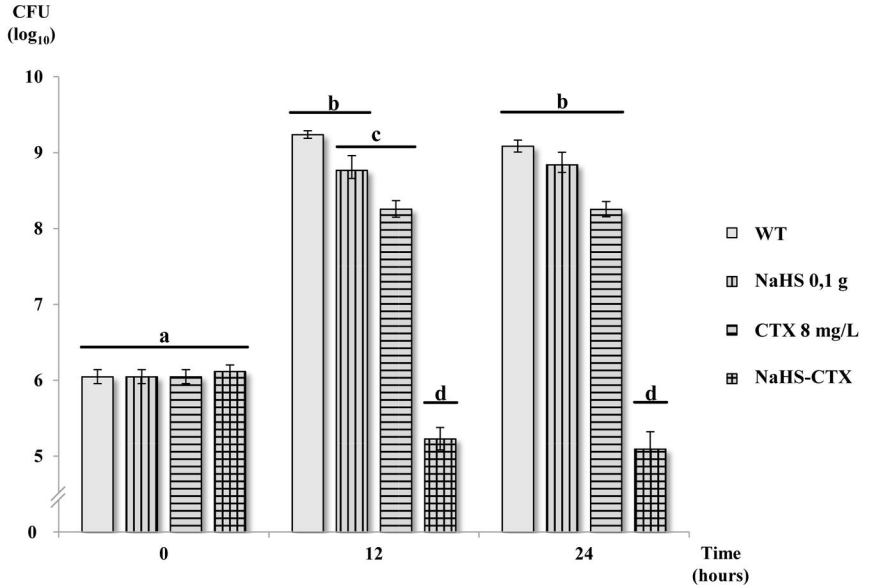
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(log10)
10
9
8
7
6
5
0
a
b
c
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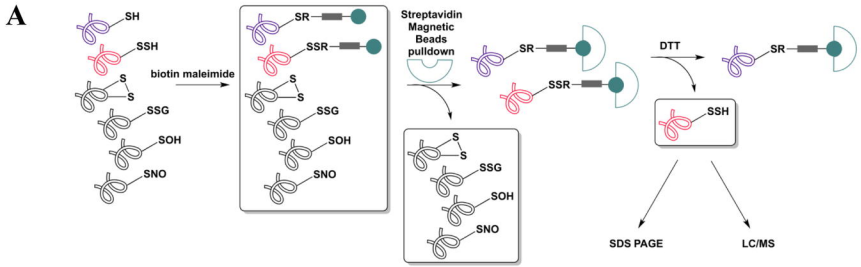
b
d
0
12
24
Time
(hours)
WT
NaHS 0,1 g
CTX 8 mg/L
NaHS-CTX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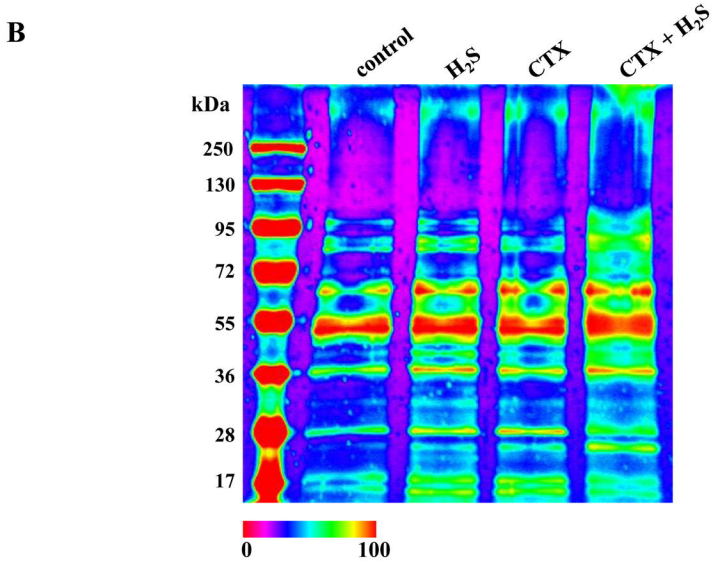
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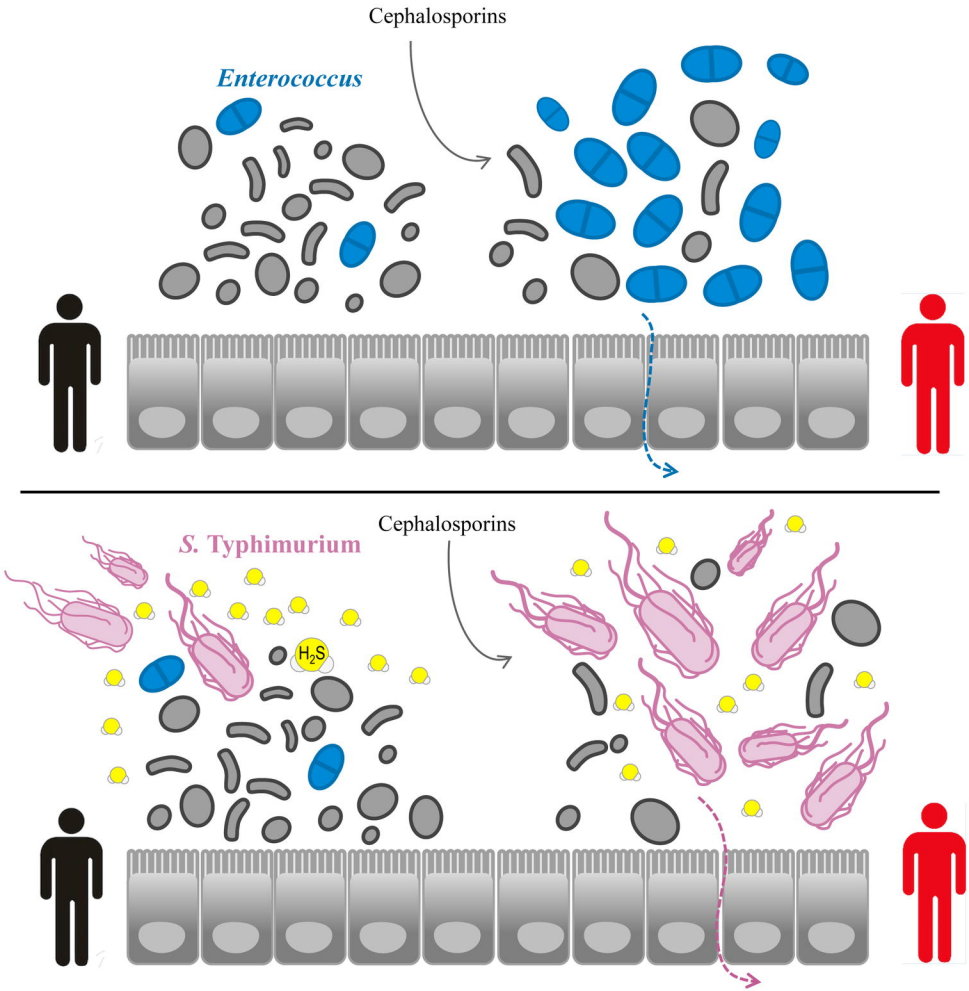
Cephalosporins
Enterococcus
Cephalosporins
S. Typhimurium
H_2S