

| Editor's Pick | Antimicrobial Chemotherapy | Short Form

Hydrogen sulfide production does not affect antibiotic resistance in *Pseudomonas aeruginosa*

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ABSTRACT Hydrogen sulfide (H₂S) has been proposed to protect bacteria from antibiotics, pointing to H₂S-producing enzymes as possible targets for the development of antibiotic adjuvants. Here, MIC assays performed with *Pseudomonas aeruginosa* mutants producing altered H₂S levels demonstrate that H₂S does not affect antibiotic resistance in this bacterium. Moreover, correlation analyses in a large collection of *P. aeruginosa* cystic fibrosis isolates argue against the protective role of H₂S from antibiotic activity during chronic lung infection.

KEYWORDS antibiotic resistance, *Pseudomonas aeruginosa*, cystic fibrosis, H₂S, antibiotic adjuvants

Multi-drug resistant (MDR) bacterial pathogens rapidly spread, and only a few novel antibacterial drugs are in the pipeline (1). This alarming situation calls for new therapies to treat MDR infections (2), including the development of adjuvants that re-empower antibiotic action (3). In this context, hydrogen sulfide (H₂S) production has attracted the attention of scientists (4, 5), as some studies demonstrated that endogenously produced H₂S reduces bacterial susceptibility to antibiotics (6–9) and that H₂S-producing enzymes are promising targets for the development of antibiotic adjuvants (10–13).

Pseudomonas aeruginosa is a primary opportunistic human pathogen, being responsible for over 300,000 deaths annually (14), and a major cause of chronic lung infection in individuals with cystic fibrosis (CF) (15). This bacterium possesses the genes coding for the H₂S-synthesizing enzymes cystathionine γ -lyase (CSE), cystathionine β -synthase (CBS) (6, 10), and mercaptopyruvate sulfurtransferase (3MST) (7), although the role of 3MST in H₂S production in *P. aeruginosa* remains unexplored. Additionally, *P. aeruginosa* utilizes sulfide:quinone oxidoreductases (SQR1 and SQR2) and the persulfide dioxygenase (PDO) to dispose of H₂S and prevent sulfide accumulation (16). To date, the actual involvement of H₂S production in *P. aeruginosa* resistance to clinically relevant antibiotics has been seldom investigated (6, 7, 10). Therefore, the potential efficacy of drugs targeting H₂S production for anti-*P. aeruginosa* therapies remains uncertain.

To assess the effect of H₂S production on *P. aeruginosa* antibiotic resistance, markerless deletion mutants producing higher or lower levels of H₂S compared to the parental strain PAO1 were generated by multi-step allelic exchange (17, 18). H₂S released by cultures of PAO1 and its isogenic mutants was quantified by using an optimized protocol based on lead acetate-soaked paper strips (6, 10, 16, 19), as detailed in Supplementary Materials and Methods. Briefly, this method relies on the staining of a lead acetate-soaked paper strip, whose intensity positively correlates with the amount of H₂S released by the bacterial culture, as confirmed by using increasing concentrations of the H₂S-donor sodium hydrosulfide (NaHS) (Fig. S1). This analysis revealed that 3MST contributes to H₂S production in *P. aeruginosa*, as the $\Delta 3mst$ mutant produced ca. 50%

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H₂S relative to PAO1 in Lysogeny Broth (LB) (Fig. 1A). Based on this evidence, the $\Delta 3syn$ mutant deleted in the three genes contributing to H₂S production in *P. aeruginosa* (i.e., *3mst*, *cbs*, and *cse*) was constructed; this mutant showed <8% residual H₂S levels compared to PAO1 (Fig. 1A). The same strategy was used to construct the $\Delta 3ox$ mutant, deleted in the three genes implicated in H₂S disposal in *P. aeruginosa* (i.e., *sqr1*, *sqr2*, and *pdo*); this mutant exhibited ca. 6.4-fold higher H₂S levels compared to PAO1 (Fig. 1A).

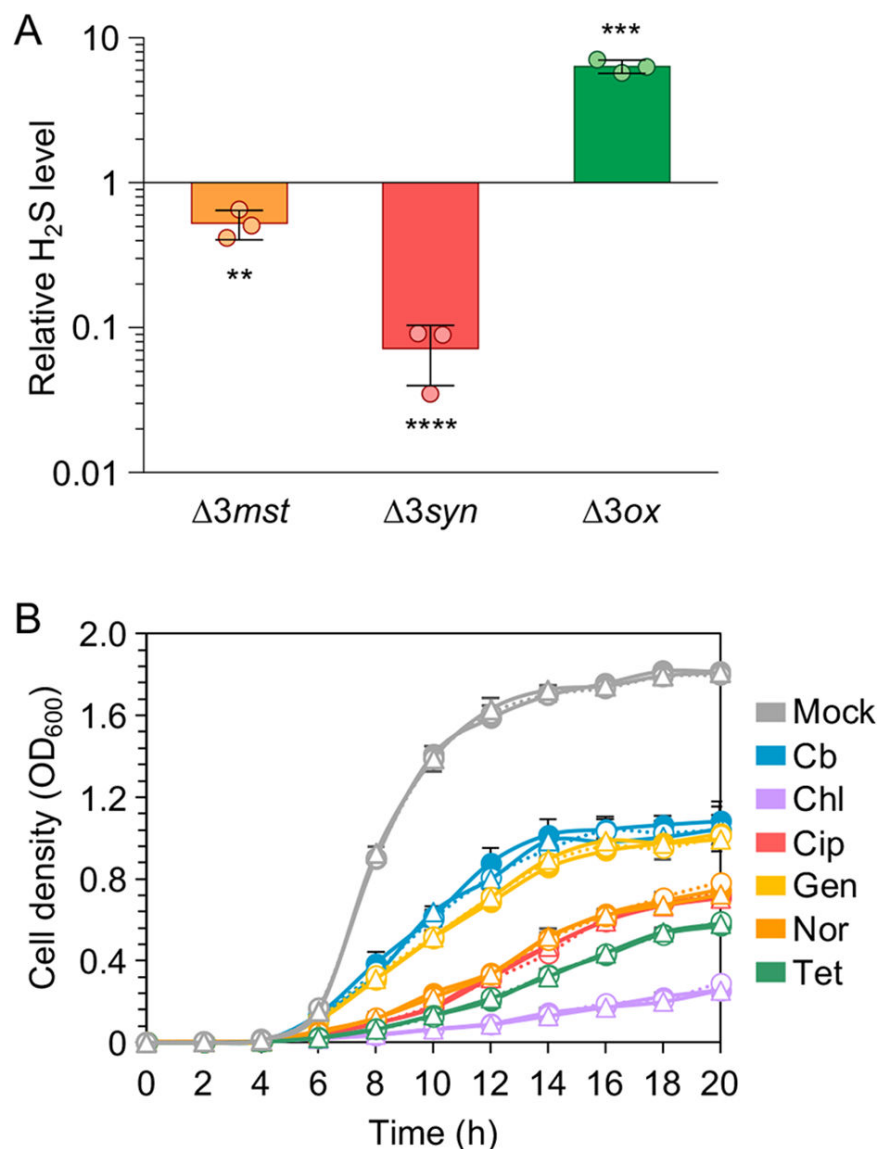


FIG 1 (A) Fold change in the H₂S levels released by the $\Delta 3mst$, $\Delta 3syn$, and $\Delta 3ox$ mutants relative to PAO1. H₂S levels were determined via densitometric analyses of lead acetate-soaked paper strips exposed for 20 hours to the bacterial cultures grown in LB. The average values of three independent experiments, each performed on eight bacterial cultures, are reported with standard deviations. Asterisks denote statistically significant differences with respect to PAO1 (** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$; unpaired t-test). (B) Growth curves of PAO1 (solid lines, full circles), $\Delta 3syn$ (dotted lines, empty circles), and $\Delta 3ox$ (solid lines, empty triangles) in LB (mock; gray lines) or in LB supplemented with the indicated antibiotics at sub-MIC concentrations (1/4 MIC). Cb, carbenicillin (blue lines); Chl, chloramphenicol (purple lines); Cip, ciprofloxacin (red lines); Gen, gentamicin (yellow lines); Nor, norfloxacin (orange lines); and Tet, tetracycline (green lines). The average values of three independent experiments are reported with standard deviations.

The standard microdilution method (20) was used to perform MIC assays for the PAO1, $\Delta 3syn$, and $\Delta 3ox$ strains grown in cation-adjusted Mueller-Hinton broth (MHB-II), LB, or tryptic soy broth supplemented with L-cysteine (TSB-cys). The addition of L-cysteine to the medium is known to increase H₂S production (6). LB and TSB-cys were also used to reproduce experimental settings used in previous studies (6, 10). Antibiotics previously tested in studies focused on H₂S production in *P. aeruginosa* (i.e., carbenicillin, chloramphenicol, ciprofloxacin, gentamicin, norfloxacin, and tetracycline) (6, 7, 10) and other antibiotics of clinical relevance for *P. aeruginosa* (i.e., colistin, meropenem, and tobramycin) were tested. As H₂S is a volatile molecule, to limit its possible leakage, MIC assays in MHB-II were also performed by sealing the microtiter plates with an adhesive plastic sheet not permeable to H₂S (Fig. S2). The $\Delta 3syn$ and $\Delta 3ox$ mutants produced lower and higher levels of H₂S relative to PAO1, respectively, also in the experimental conditions used for the MIC assays (Fig. S3). MIC values of all the tested antibiotics were the same for PAO1, $\Delta 3syn$, and $\Delta 3ox$ in all conditions (Table 1). Notably, the PAO1, $\Delta 3syn$, and $\Delta 3ox$ strains showed comparable growth curves when treated with sub-MIC concentrations of previously tested antibiotics in LB (Fig. 1B) or in LB supplemented with L-cysteine or NaHS (Fig. S4). This demonstrates that H₂S levels do not affect *P. aeruginosa* growth kinetics in the presence of antibiotics.

Resistance to many clinically relevant classes of antibiotics is frequently observed in *P. aeruginosa* strains isolated from CF patients with chronic lung infection (21, 22). To assess a possible correlation between H₂S production and antibiotic resistance in clinical isolates, we quantified the H₂S levels produced by 100 clinical isolates of *P. aeruginosa* from CF lungs with distinct antibiotic resistance profiles (Fig. 2A) (23, 24). This analysis revealed that H₂S levels are lower in resistant and MDR isolates relative to sensitive ones (Fig. 2B) and that a decrease in H₂S production parallels the progression of chronic infection (Fig. 2C). Hence, high levels of H₂S production appear to be counter-selected during *in vivo* infection in the CF lung, despite antibiotic resistance increases.

Overall, our data argue against the role of H₂S in conferring antibiotic resistance during CF lung infection. While it is possible that specific host-associated stimuli could boost *P. aeruginosa* H₂S production to protective levels *in vivo*, this possibility is discredited by the evidence that H₂S does not protect *P. aeruginosa* from antibiotics even when produced at high levels, as observed in the $\Delta 3ox$ mutant.

Our data do not support the protective role of H₂S against antibiotics claimed for *P. aeruginosa* in previous studies (6, 7, 10). Similar discrepancies have been reported for *Staphylococcus aureus*; while some studies reported that H₂S production confers antibiotic resistance to this bacterium (6, 10), a contrasting study indicated that endogenous H₂S levels are not sufficient to protect *S. aureus* from aminoglycosides

TABLE 1 MIC values for PAO1 and its isogenic mutants $\Delta 3syn$ and $\Delta 3ox$

Medium	Strain	MIC ($\mu\text{g/mL}$) ^a								
		Cb	Chl	Cip	Col	Gen	Mer	Nor	Tet	Tob
MHB-II	PAO1	128	64	0.25	1	4	1	1	32	2
	$\Delta 3syn$	128	64	0.25	1	4	1	1	32	2
	$\Delta 3ox$	128	64	0.25	1	4	1	1	32	2
MHB-II wps	PAO1	128	64	0.25	1	4	1	1	32	2
	$\Delta 3syn$	128	64	0.25	1	4	1	1	32	2
	$\Delta 3ox$	128	64	0.25	1	4	1	1	32	2
LB	PAO1	128	64	0.25	1	4	1	1	16	2
	$\Delta 3syn$	128	64	0.25	1	4	1	1	16	2
	$\Delta 3ox$	128	64	0.25	1	4	1	1	16	2
TSB-cys	PAO1	128	64	0.125	1	4	1	0.5	32	1
	$\Delta 3syn$	128	64	0.125	1	4	1	0.5	32	1
	$\Delta 3ox$	128	64	0.125	1	4	1	0.5	32	1

^aCb, carbenicillin; Chl, chloramphenicol; Cip, ciprofloxacin; Col, colistin; Gen, gentamicin; Mer, meropenem; Nor, norfloxacin; Tet, tetracycline; Tob, tobramycin; MHB-II wps, MHB-II sealed with plastic sheet; and TSB-cys, tryptic soy broth supplemented with 200 μM L-cysteine.

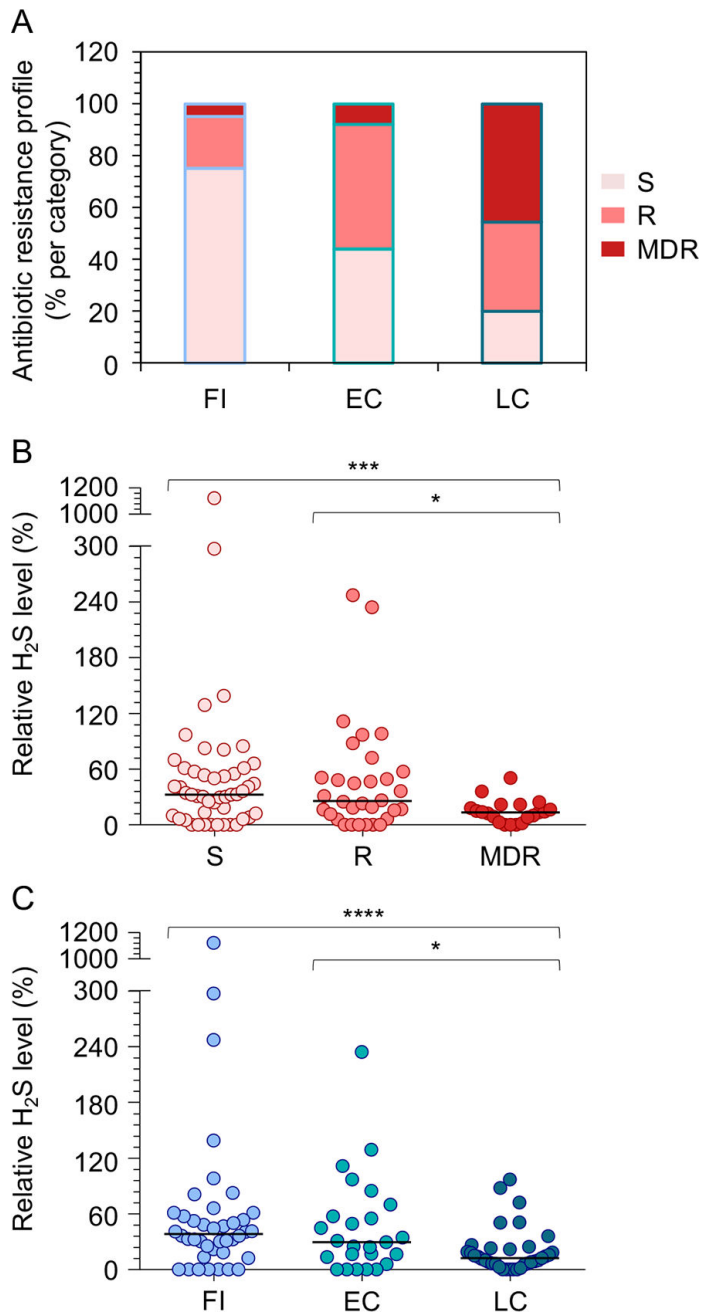


FIG 2 (A) Antibiotic resistance pattern of 100 CF isolates categorized by the duration of the lung infection (23, 24). FI, first isolate ($n = 40$); EC, early chronic ($n = 25$); LC, late chronic ($n = 35$); S, susceptible to all antibiotics tested ($n = 48$); R, resistant to one or two antibiotics of different classes ($n = 32$); and MDR, multi-drug resistant (non-susceptible to at least one agent in three or more classes of antibiotics) ($n = 20$). (B) Relative H₂S levels released in LB by the CF isolates grouped by antibiotic resistance pattern. (C) Relative H₂S levels produced in LB by the CF isolates grouped by the duration of the chronic lung infection. For panels B and C, each circle represents the average of three independent experiments for a given strain; the percentages refer to the H₂S level measured in PAO1 in LB, considered as 100%. Horizontal lines indicate median values. Asterisks denote statistically significant differences between groups (* $P < 0.05$; *** $P < 0.001$; and **** $P < 0.0001$; Kolmogorov-Smirnov test).

and that exogenous provision of H₂S decreases *S. aureus* resistance to non-aminoglycoside antibiotics (25). H₂S decreases antibiotic resistance in *Acinetobacter baumannii* too (26). Interestingly, a mutant of *Fusobacterium nucleatum* substantially deficient in H₂S

production gained significant sensitivity to nalidixic acid and resistance to kanamycin (9). By providing evidence that H₂S production is not a defense mechanism against antibiotics in *P. aeruginosa*, our data support the notion that H₂S does not act as a protective molecule in all bacterial species.

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ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental figures and tables and additional experimental details (AAC00075-24-s0001.pdf). Figure S1 (H₂S calibration curve), Figure S2 (The plastic sheets used in this study are not permeable to H₂S), Figure S3 (H₂S production under conditions used for MIC assays), Figure S4 (Growth curves in the presence of antibiotics at sub-MIC concentration), Table S1 (Bacterial strains used in this study), Table S2 (Plasmids used in this study), Table S3 (Oligonucleotides used in this study), and detailed materials and methods.

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