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

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ABSTRACT Hydrogen sulfide (H_2S) has been proposed to protect bacteria from antibiotics, pointing to H_2S-producing enzymes as possible targets for the development of antibiotic adjuvants. Here, MIC assays performed with *Pseudomonas aeruginosa* mutants producing altered H_2S levels demonstrate that H_2S 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 H_2S from antibiotic activity during chronic lung infection.

KEYWORDS antibiotic resistance, *Pseudomonas aeruginosa*, cystic fibrosis, H_2S, 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 (H_2S) production has attracted the attention of scientists (4, 5), as some studies demonstrated that endogenously produced H_2S reduces bacterial susceptibility to antibiotics (6–9) and that H_2S-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 H_2S-synthesizing enzymes cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) (6, 10), and mercaptopyruvate sulfurtransferase (3MST) (7), although the role of 3MST in H_2S production in *P. aeruginosa* remains unexplored. Additionally, *P. aeruginosa* utilizes sulfide:quinone oxidoreductases (SQR1 and SQR2) and the persulfide dioxygenase (PDO) to dispose of H_2S and prevent sulfide accumulation (16). To date, the actual involvement of H_2S production in *P. aeruginosa* resistance to clinically relevant antibiotics has been seldom investigated (6, 7, 10). Therefore, the potential efficacy of drugs targeting H_2S production for anti-*P. aeruginosa* therapies remains uncertain.

To assess the effect of H_2S production on *P. aeruginosa* antibiotic resistance, markerless deletion mutants producing higher or lower levels of H_2S compared to the parental strain PAO1 were generated by multi-step allelic exchange (17, 18). H_2S 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 H_2S released by the bacterial culture, as confirmed by using increasing concentrations of the H_2S-donor sodium hydrosulfide (NaHS) (Fig. S1). This analysis revealed that 3MST contributes to H_2S production in *P. aeruginosa*, as the Δ*3mst* mutant produced ca. 50%

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H_2S relative to PAO1 in Lysogeny Broth (LB) (Fig. 1A). Based on this evidence, the Δ3*syn* mutant deleted in the three genes contributing to H_2S production in *P. aeruginosa* (i.e., *3mst*, *cbs*, and *cse*) was constructed; this mutant showed <8% residual H_2S levels compared to PAO1 (Fig. 1A). The same strategy was used to construct the Δ3*ox* mutant, deleted in the three genes implicated in H_2S disposal in *P. aeruginosa* (i.e., *sqr1*, *sqr2*, and *pdo*); this mutant exhibited ca. 6.4-fold higher H_2S levels compared to PAO1 (Fig. 1A).

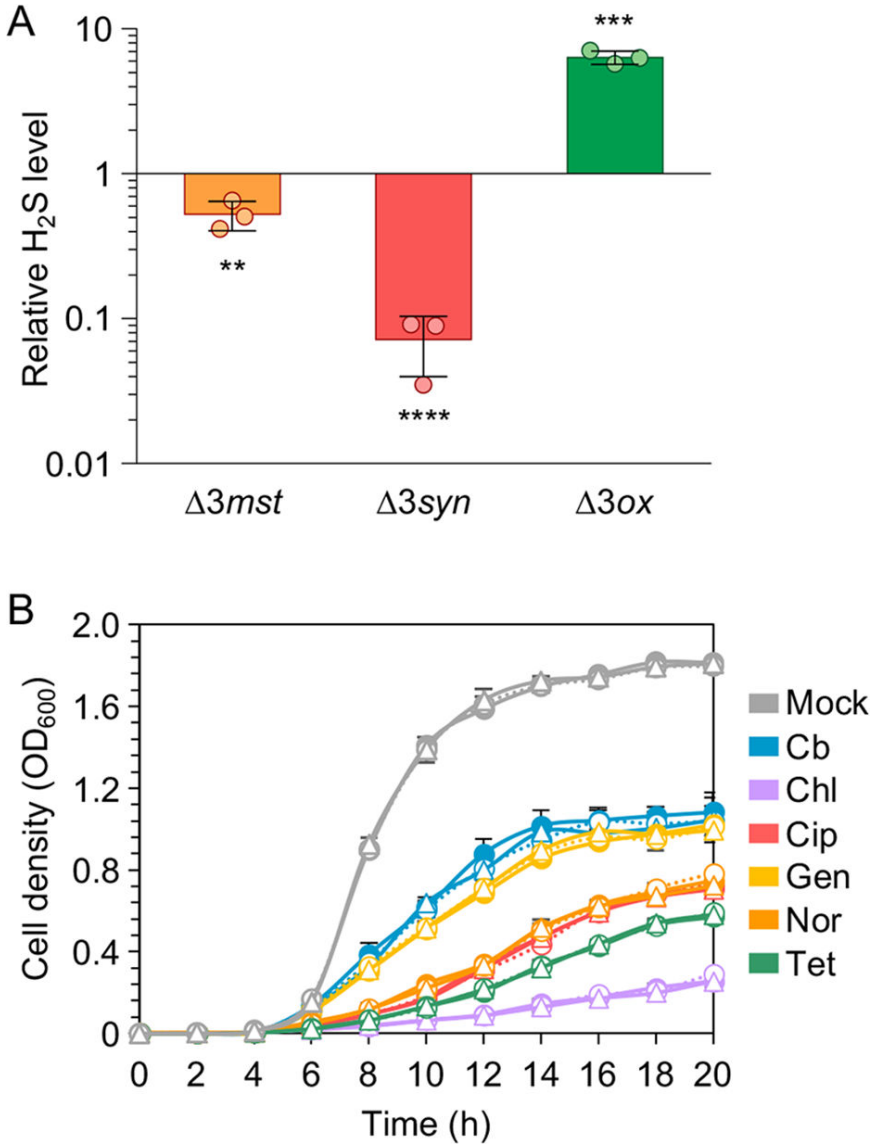

FIG 1 (A) Fold change in the H_2S levels released by the Δ3*mst*, Δ3*syn*, and Δ3*ox* mutants relative to PAO1. H_2S levels were determined via densitometric analyses of lead acetate-soaked paper strips exposed for 20 hours to the bacterial cultures grown in LB. The average values of three independent 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), Δ3*syn* (dotted lines, empty circles), and Δ3*ox* (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, Δ3*syn,* and Δ3*ox* 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 H_2S 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 H_2S 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 H_2S 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 H_2S (Fig. S2). The Δ3*syn* and Δ3*ox* mutants produced lower and higher levels of H_2S 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, Δ3*syn,* and Δ3*ox* in all conditions (Table 1). Notably, the PAO1, Δ3*syn,* and Δ3*ox* 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 H_2S 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 H_2S production and antibiotic resistance in clinical isolates, we quantified the H_2S 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 H_2S levels are lower in resistant and MDR isolates relative to sensitive ones (Fig. 2B) and that a decrease in H_2S production parallels the progression of chronic infection (Fig. 2C). Hence, high levels of H_2S 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 H_2S in conferring antibiotic resistance during CF lung infection. While it is possible that specific host-associated stimuli could boost *P. aeruginosa* H_2S production to protective levels *in vivo*, this possibility is discredited by the evidence that H_2S does not protect *P. aeruginosa* from antibiotics even when produced at high levels, as observed in the Δ3*ox* mutant.

Our data do not support the protective role of H_2S 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 H_2S production confers antibiotic resistance to this bacterium (6, 10), a contrasting study indicated that endogenous H_2S levels are not sufficient to protect *S. aureus* from aminoglycosides

TABLE 1 MIC values for PAO1 and its isogenic mutants Δ3*syn* and Δ3*ox*

		MIC (μg/mL)[a]								
Medium	**Strain**	**Cb**	**Chl**	**Cip**	**Col**	**Gen**	**Mer**	**Nor**	**Tet**	**Tob**
MHB-II	PAO1	128	64	0.25	1	4	1	1	32	2
	Δ3*syn*	128	64	0.25	1	4	1	1	32	2
	Δ3*ox*	128	64	0.25	1	4	1	1	32	2
MHB-II wps	PAO1	128	64	0.25	1	4	1	1	32	2
	Δ3*syn*	128	64	0.25	1	4	1	1	32	2
	Δ3*ox*	128	64	0.25	1	4	1	1	32	2
LB	PAO1	128	64	0.25	1	4	1	1	16	2
	Δ3*syn*	128	64	0.25	1	4	1	1	16	2
	Δ3*ox*	128	64	0.25	1	4	1	1	16	2
TSB-cys	PAO1	128	64	0.125	1	4	1	0.5	32	1
	Δ3*syn*	128	64	0.125	1	4	1	0.5	32	1
	Δ3*ox*	128	64	0.125	1	4	1	0.5	32	1

[a]Cb, 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.

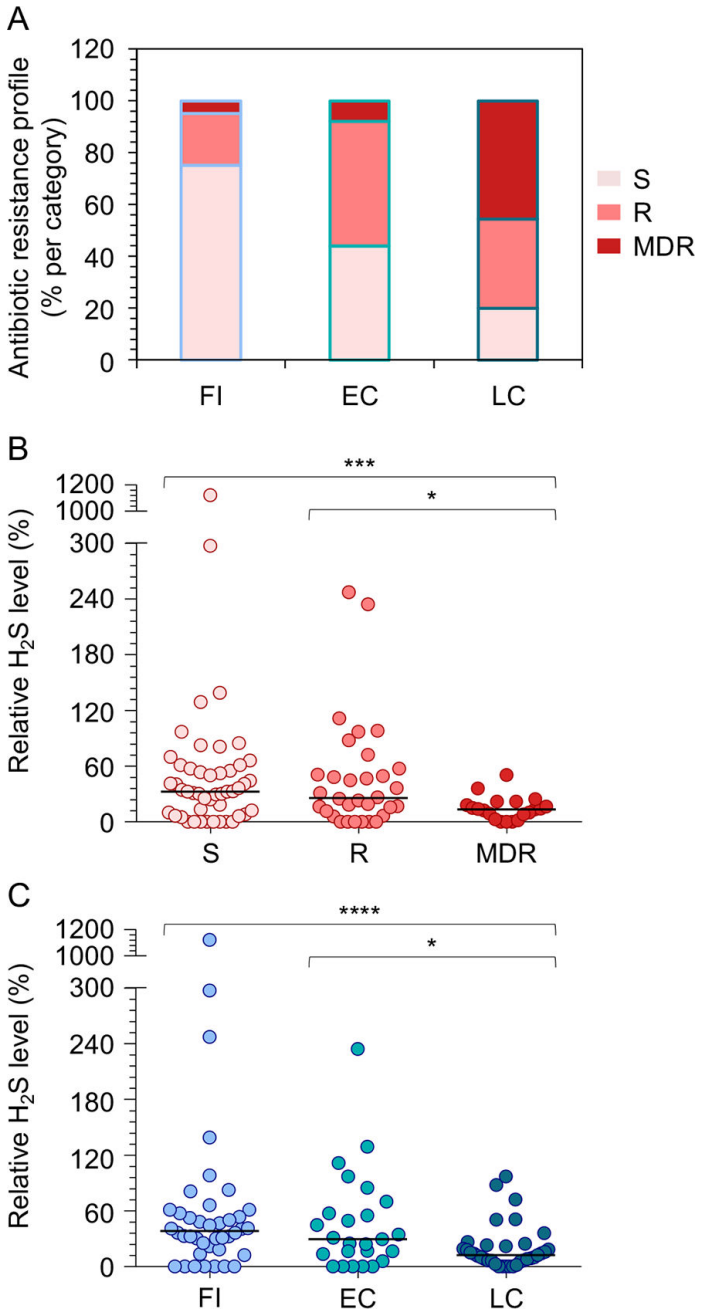

FIG 2 (A) Antibiotic resistance pattern of 100 CF isolates categorized by the duration of the lung infection (23, 24). FI, first isolate (n = 40); EC, early chronic (n = 25); LC, late chronic (n = 35); S, susceptible to all antibiotics tested (n = 48); R, resistant to one or two antibiotics of different 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 H_2S levels released in LB by the CF isolates grouped by antibiotic resistance pattern. (C) Relative H_2S 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 H_2S 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 H_2S decreases *S. aureus* resistance to non-aminoglycoside antibiotics (25). H_2S decreases antibiotic resistance in *Acinetobacter baumannii* too (26). Interestingly, a mutant of *Fusobacterium nucleatum* substantially deficient in H_2S

production gained significant sensitivity to nalidixic acid and resistance to kanamycin (9). By providing evidence that H_2S production is not a defense mechanism against antibiotics in *P. aeruginosa,* our data support the notion that H_2S 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 (H_2S calibration curve), Figure S2 (The plastic sheets used in this study are not permeable to H_2S), Figure S3 (H_2S 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.

REFERENCES

1. Darby EM, Trampari E, Siasat P, Gaya MS, Alav I, Webber MA, Blair JMA. 2023. Molecular mechanisms of antibiotic resistance revisited. Nat Rev Microbiol 21:280–295. https://doi.org/10.1038/s41579-022-00820-y
2. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, Pulcini C, Kahlmeter G, Kluytmans J, Carmeli Y, Ouellette M, Outterson K, Patel J, Cavaleri M, Cox EM, Houchens CR, Grayson ML, Hansen P, Singh N, Theuretzbacher U, Magrini N, WHO Pathogens Priority List Working Group. 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis 18:318–327. https://doi.org/10.1016/S1473-3099(17)30753-3
3. Gill EE, Franco OL, Hancock REW. 2015. Antibiotic adjuvants: diverse strategies for controlling drug-resistant pathogens. Chem Biol Drug Des 85:56–78. https://doi.org/10.1111/cbdd.12478
4. Luhachack L, Nudler E. 2014. Bacterial gasotransmitters: an innate defense against antibiotics. Curr Opin Microbiol 21:13–17. https://doi.org/10.1016/j.mib.2014.06.017
5. Fang D, Wang Z, Liu Y. 2023. Cystathionine γ-lyase: the Achilles heel of bacterial defense systems. Int J Antimicrob Agents 62:106845. https://doi.org/10.1016/j.ijantimicag.2023.106845
6. Shatalin K, Shatalina E, Mironov A, Nudler E. 2011. H_2S: a universal defense against antibiotics in bacteria. Science 334:986–990. https://doi.org/10.1126/science.1209855
7. Xuan G, Lü C, Xu H, Chen Z, Li K, Liu H, Liu H, Xia Y, Xun L. 2020. Sulfane sulfur is an intrinsic signal activating MexR-regulated antibiotic resistance in *Pseudomonas aeruginosa*. Mol Microbiol 114:1038–1048. https://doi.org/10.1111/mmi.14593
8. Kunota TTR, Rahman M, Truebody BE, Mackenzie JS, Saini V, Lamprecht DA, Adamson JH, Sevalkar RR, Lancaster JR, Berney M, Glasgow JN, Steyn AJC. 2021. *Mycobacterium tuberculosis* H_2S functions as a sink to modulate central metabolism, bioenergetics, and drug susceptibility. Antioxidants (Basel) 10:1285. https://doi.org/10.3390/antiox10081285
9. Chen YW, Camacho MI, Chen Y, Bhat AH, Chang C, Peluso EA, Wu C, Das A, Ton-That H. 2022. Genetic determinants of hydrogen sulfide biosynthesis in *Fusobacterium nucleatum* are required for bacterial fitness, antibiotic sensitivity, and virulence. mBio 13:e0193622. https://doi.org/10.1128/mbio.01936-22
10. Shatalin K, Nuthanakanti A, Kaushik A, Shishov D, Peselis A, Shamovsky I, Pani B, Lechpammer M, Vasilyev N, Shatalina E, Rebatchouk D, Mironov A, Fedichev P, Serganov A, Nudler E. 2021. Inhibitors of bacterial H_2S biogenesis targeting antibiotic resistance and tolerance. Science 372:1169–1175. https://doi.org/10.1126/science.abd8377
11. Kuzovlev AS, Zybalov MD, Golovin AV, Gureev MA, Kasatkina MA, Biryukov MV, Belik AR, Silonov SA, Yunin MA, Zigangirova NA, Reshetnikov VV, Isakova YE, Porozov YB, Ivanov RA. 2023. Naphthyl-substituted indole and pyrrole carboxylic acids as effective antibiotic potentiators-inhibitors of bacterial cystathionine γ-lyase. Int J Mol Sci 24:16331. https://doi.org/10.3390/ijms242216331
12. Croppi G, Zhou Y, Yang R, Bian Y, Zhao M, Hu Y, Ruan BH, Yu J, Wu F. 2020. Discovery of an inhibitor for bacterial 3-mercaptopyruvate sulfurtransferase that synergistically controls bacterial survival. Cell Chem Biol 27:1483–1499. https://doi.org/10.1016/j.chembiol.2020.10.012
13. Seregina TA, Lobanov KV, Shakulov RS, Mironov AS. 2022. Enhancement of the bactericidal effect of antibiotics by inhibition of enzymes involved in production of hydrogen sulfide in bacteria. Mol Biol 56:638–648. https://doi.org/10.1134/S0026893322050120
14. GBD 2019 Antimicrobial Resistance Collaborators. 2022. Global mortality associated with 33 bacterial pathogens in 2019: a systematic analysis for the global burden of disease study 2019. Lancet 400:2221–2248. https://doi.org/10.1016/S0140-6736(22)02185-7
15. Moradali MF, Ghods S, Rehm BHA. 2017. *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. Front Cell Infect Microbiol 7:39. https://doi.org/10.3389/fcimb.2017.00039
16. Xia Y, Lü C, Hou N, Xin Y, Liu J, Liu H, Xun L. 2017. Sulfide production and oxidation by heterotrophic bacteria under aerobic conditions. ISME J 11:2754–2766. https://doi.org/10.1038/ismej.2017.125
17. Milton DL, O'Toole R, Horstedt P, Wolf-Watz H. 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum*. J Bacteriol 178:1310–1319. https://doi.org/10.1128/jb.178.5.1310-1319.1996
18. Letizia M, Mellini M, Fortuna A, Visca P, Imperi F, Leoni L, Rampioni G. 2022. PqsE expands and differentially modulates the RhlR quorum sensing regulon in *Pseudomonas aeruginosa*. Microbiol Spectr 10:e0096122. https://doi.org/10.1128/spectrum.00961-22
19. Forbes BA, Sahm DF, Weissfeld AS. 1998. Bailey and Scott's diagnostic microbiology. 10th ed. Mosby Inc.

20. Clinical and Laboratory Standards Institute. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 9th ed. CLSI (Approved standard M07- A9), Wayne PA.
21. Lister PD, Wolter DJ, Hanson ND. 2009. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev 22:582–610. https://doi.org/10.1128/CMR.00040-09
22. Marvig RL, Sommer LM, Molin S, Johansen HK. 2015. Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. Nat Genet 47:57–64. https://doi.org/10.1038/ng.3148
23. Imperi F, Fiscarelli EV, Visaggio D, Leoni L, Visca P. 2019. Activity and impact on resistance development of two antivirulence fluoropyrimidine drugs in *Pseudomonas aeruginosa*. Front Cell Infect Microbiol 9:49. https://doi.org/10.3389/fcimb.2019.00049
24. Collalto D, Giallonardi G, Fortuna A, Meneghini C, Fiscarelli E, Visca P, Imperi F, Rampioni G, Leoni L. 2022. *In vitro* activity of antivirulence drugs targeting the *las* or *pqs* quorum sensing against cystic fibrosis *Pseudomonas aeruginosa* isolates. Front Microbiol 13:845231. https://doi.org/10.3389/fmicb.2022.845231
25. Weikum J, Ritzmann N, Jelden N, Klöckner A, Herkersdorf S, Josten M, Sahl HG, Grein F. 2018. Sulfide protects *Staphylococcus aureus* from aminoglycoside antibiotics but cannot be regarded as a general defense mechanism against antibiotics. Antimicrob Agents Chemother 62:e00602-18. https://doi.org/10.1128/AAC.00602-18
26. Ng SY, Ong KX, Surendran ST, Sinha A, Lai JJH, Chen J, Liang J, Tay LKS, Cui L, Loo HL, Ho P, Han J, Moreira W. 2020. Hydrogen sulfide sensitizes *Acinetobacter baumannii* to killing by antibiotics. Front Microbiol 11:1875. https://doi.org/10.3389/fmicb.2020.01875