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The Terminal Oxidase Cytochrome *bd* Promotes Sulfide-resistant Bacterial Respiration and Growth

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Hydrogen sulfide (H_2S) impairs mitochondrial respiration by potently inhibiting the heme-copper cytochrome *c* oxidase. Since many prokaryotes, including *Escherichia (E.) coli*, generate H_2S and encounter high H_2S levels particularly in the human gut, herein we tested whether bacteria can sustain sulfide-resistant O_2-dependent respiration. *E. coli* has three respiratory oxidases, the cyanide-sensitive heme-copper bo_3 enzyme and two *bd* oxidases much less sensitive to cyanide. Working on the isolated enzymes, we found that, whereas the bo_3 oxidase is inhibited by sulfide with half-maximal inhibitory concentration $IC_{50} = 1.1 \pm 0.1\,\mu M$, under identical experimental conditions both *bd* oxidases are insensitive to sulfide up to 58 μM. In *E. coli* respiratory mutants, both O_2-consumption and aerobic growth proved to be severely impaired by sulfide when respiration was sustained by the bo_3 oxidase alone, but unaffected by $\leq$200 μM sulfide when either *bd* enzyme acted as the only terminal oxidase. Accordingly, wild-type *E. coli* showed sulfide-insensitive respiration and growth under conditions favouring the expression of *bd* oxidases. In all tested conditions, cyanide mimicked the functional effect of sulfide on bacterial respiration. We conclude that *bd* oxidases promote sulfide-resistant O_2-consumption and growth in *E. coli* and possibly other bacteria. The impact of this discovery is discussed.

Along with nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide (H_2S) has been recognized as an important gaseous signalling molecule, playing a major role in human (patho)physiology[1]. Like NO and CO, H_2S is a key regulator of many physiological processes in the cardiovascular, nervous, respiratory and gastrointestinal systems, among others. While exerting beneficial physiological effects at lower levels, at higher concentrations H_2S can cause detrimental effects. In eukaryotes, depending on its concentration, H_2S can have opposite effects on respiration (reviewed in[2]): at nanomolar concentrations it can sustain energy metabolism both as a substrate for the mitochondrial respiratory chain and as a vasodilator favouring O_2 supply, whereas at higher levels it impairs cellular respiration via direct binding to and inhibition of mitochondrial cytochrome *c* oxidase (mtCcOX) (see[3] and references therein). Sulfide inhibition of mtCcOX is very effective ($K_i = 0.2$–$0.45\,\mu M$ at pH = 7.4[3,4]), leading to dissipation of the mitochondrial membrane potential, consequent arrest of aerobic ATP production and eventually cell death[2].

In mammalian tissues, H_2S is enzymatically produced by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and via the combined action of 3-mercaptopyruvate sulfurtransferase (3-MST) and cysteine aminotransferase (reviewed in[5]). At variance from other compartments in the human body, in the intestinal lumen H_2S is also generated by the gut microbiota through bacterial amino acid metabolism and *via* dissimilatory sulfate reduction by 'sulfate-reducing bacteria' (SRB)[6]. H_2S levels in the gut are therefore high. Whereas the total sulfide pool content in the colon is around one millimolar[7], the concentration of free H_2S in the intestinal lumen was

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reported to be ca. 40–60 μM, as estimated by direct measurement of the gas in the rat cecum[8,9] and analysis of human faecal samples[10].

E. coli is a ubiquitous member of the human gut microbiota, with more than one strain commonly colonizing the large intestine at the same time. Since *E. coli*, like the other microorganisms inhabiting the gut, lives in a particularly H_2S-enriched microaerobic niche, the question arises as to whether this microorganism can accomplish O_2-dependent respiration without being inhibited by H_2S. The *E. coli* respiratory chain possesses three terminal oxygen reductases, utilizing quinols as reducing substrates: the cyanide-sensitive cytochrome bo_3 enzyme and the *bd*-I and *bd*-II oxidases, much less sensitive to cyanide[11,12]. Cytochrome bo_3 belongs to the superfamily of heme–copper oxygen reductases that includes mtCcOX. The enzyme contains three redox-active metal centres: the low-spin heme *b* involved in quinol oxidation and a binuclear site composed of heme o_3 and Cu_B, where O_2 reduction to water takes place. On the contrary, *bd*-I and *bd*-II are cytochrome *bd*-type O_2-reductases phylogenetically unrelated to heme–copper oxidases[12]. They have no copper, but contain three hemes: the low-spin heme b_{558} (the primary electron acceptor from the quinol), and the two high-spin hemes b_{595} and *d* (possibly forming a di-heme site for O_2 reduction, see[12] and references therein). Cytochrome bo_3 predominates in *E. coli* under high aeration, whereas O_2-limiting conditions such as those found in the human gut stimulate the expression of the cytochromes *bd*-I and *bd*-II[13–15]. The three *E. coli* terminal oxidases all generate a proton motive force, but cytochrome bo_3 is the only one able to pump protons, thus being twice as effective as *bd*-type cytochromes in terms of energy transduction[16]. Besides its role in bacterial energy metabolism, cytochrome *bd*-I was suggested to serve other physiological functions, being implicated in the bacterial response to oxidative and nitrosative stress[17–20].

In this work, we examined the effect of sulfide on the O_2 reductase activity of the three terminal oxidases of *E. coli* and tested the ability of these enzymes to sustain bacterial growth and O_2 consumption in the presence of sulfide.

Results

Effect of NaHS on isolated *E. coli* terminal oxidases. The effect of sulfide on the O_2 reductase activity of the *E. coli* respiratory oxidases, cytochromes bo_3, *bd*-I and *bd*-II, was initially investigated testing the ability of each purified oxidase to consume O_2 before and after addition of the sulfide donor NaHS. In these assays, O_2 consumption was measured in the presence of dithiothreitol (DTT) and 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone (Q_1) as the reducing system. As shown in Fig. 1A, NaHS (~7 μM) rapidly and effectively inhibits the O_2 reductase activity of the isolated cytochrome bo_3. The enzyme is inhibited with an apparent half-maximal inhibitory concentration $IC_{50} = 1.1 \pm 0.1$ μM (Fig. 2). The inhibition of cytochrome bo_3 is fully reversible. A rapid and complete recovery of the O_2 reductase activity of the isolated enzyme was observed, when sulfide was quickly removed from solution by addition of an excess of *O*-acetyl-L-serine (OAS) and catalytic amounts of the sulfide-consuming *O*-acetylserine sulfhydrylase enzyme from *Entamoeba histolytica* (*Eh*OASS, Fig. 1A). Sulfide consumption by *Eh*OASS in the presence of OAS was assessed independently using a H_2S-selective electrode (Figure S1). Notably, while being an effective inhibitor of *E. coli* cytochrome bo_3, NaHS proved to be unable to inhibit the two *E. coli bd*-type oxidases. Addition of NaHS, even at high concentration (58 μM), did not alter the O_2 consumption catalyzed by the *bd*-I or *bd*-II enzyme in the presence of DTT and Q_1 (Fig. 1A). No O_2 consumption stimulation by the OAS/*Eh*OASS sulfide-scavenging system was observed in control oxygraphic experiments carried out in the absence or presence of the isolated oxidases (not shown).

Effect of NaHS on *E. coli* respiration. The striking results obtained with the isolated enzymes prompted us to explore the effect of sulfide on *E. coli* cell respiration. To this end, we investigated aerobic cultures of *E. coli* (see Methods for details) and tested the effect of NaHS on cell respiration along cell growth, i.e., at increasing cell density. We initially assayed three mutant strains each expressing a single terminal oxidase (bo_3, *bd*-I or *bd*-II). The results were remarkably similar to those obtained with the isolated enzymes. O_2 consumption by *E. coli* cells expressing solely cytochrome bo_3 was quickly and fully inhibited upon addition of 50 μM NaHS (Fig. 1B). As observed with the isolated bo_3 enzyme, the inhibition was promptly and fully restored upon sulfide depletion by the *Eh*OASS/OAS system (Fig. 1B). In contrast, no inhibition was observed following the addition of 50 μM NaHS to *E. coli* cells expressing either *bd*-I or *bd*-II as the only terminal oxidase (Fig. 1B). The results on the three mutant strains proved to be independent of the density at which cells were collected and assayed (Fig. 3, top panel). Similarly to NaHS, cyanide (50 μM) almost completely abolished O_2-consumption in *E. coli* cells expressing only the bo_3 oxidase, whereas it was essentially ineffective when respiration was sustained by either *bd* oxidase (Fig. 3, bottom panel).

The effect of NaHS on respiration of the wild-type strain was assessed in the same way. Namely, we investigated aerobic cultures in which a change in oxidase expression from cytochrome bo_3 to the cytochromes of the *bd*-type is expected to take place along cell growth, following a progressive reduction in O_2 availability in the medium[21,22]. Accordingly, when cells were assayed in an early phase of the culture ($OD_{600} < 0.7$), most of respiration (65–70%) proved to be sensitive to NaHS or cyanide (both at 50 μM, Fig. 3). In contrast, with cell growth bacterial O_2-consumption became progressively less sensitive to sulfide inhibition and, in a late phase of the culture ($OD_{600} > 2.5$), NaHS or cyanide caused only marginal effects on respiration (Fig. 3).

Altogether these results show that, unlike the heme-copper bo_3 oxidase, *E. coli bd* oxidases enable O_2-dependent respiration in the presence of sulfide.

Effect of NaHS on *E. coli* cell growth. The lack of sulfide inhibition of cytochromes *bd*-I and *bd*-II, as opposed to the high sensitivity displayed by the bo_3 oxidase, prompted us to test whether the *bd*-type oxidases, besides enabling respiration, promote *E. coli* cell growth in the presence of sulfide. We investigated the effect of sulfide on the growth of both the wild-type and the three respiratory mutant strains. Following the addition of

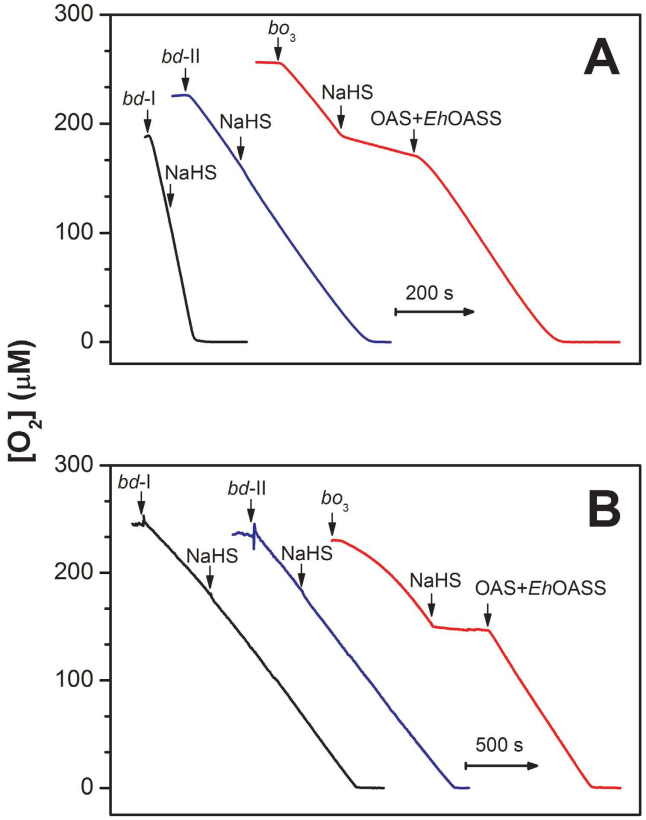

Figure 1. Effect of NaHS on *E. coli* terminal oxidases. (**A**) O_2 reductase activity of the isolated cytochromes *bd*-I (20 nM), *bd*-II (2.5 nM) and bo_3 (6 nM) as measured at 25 °C in the presence of DTT (10 mM) and Q_1 (0.25 mM). O_2 consumption rates measured prior to NaHS addition (mean ± standard deviation, n = 3): 1.62 ± 0.07 μM O_2/s (*bd*-I); 0.54 ± 0.02 μM O_2/s (*bd*-II) and 0.46 ± 0.02 μM O_2/s (bo_3). O_2 consumption by cytochrome bo_3 is rapidly inhibited (~85%) by 7.2 μM NaHS, to be quickly and completely restored upon removal of sulfide from solution following the addition of 200 μM OAS and 216 nM *Eh*OASS. On the contrary, NaHS (58 μM) does not affect the oxidase activity of either cytochrome *bd*-I or cytochrome *bd*-II. (**B**) O_2 consumption by cell suspensions of the mutant strains expressing cytochrome *bd*-I (400 μl cells with OD_{600} = 1.85), cytochrome *bd*-II (600 μl cells with OD_{600} = 1.17) or cytochrome bo_3 (200 μl cells with OD_{600} = 2.45), as the only terminal oxidase. O_2 consumption rates measured prior to NaHS addition (mean ± standard deviation, n = 3): 0.20 ± 0.07 μM O_2/s (*bd*-I); 0.18 ± 0.02 μM O_2/s (*bd*-II) and 0.19 ± 0.02 μM O_2/s (bo_3). When sustained solely by cytochrome bo_3, cell respiration is rapidly inhibited by 50 μM NaHS, to be quickly and completely restored following sulfide removal on addition of OAS (200 μM) and *Eh*OASS (216 nM). No inhibition is observed following the addition of NaHS (50 μM), when respiration is sustained by the only cytochrome *bd*-I or *bd*-II.

200 μM NaHS, the wild-type strain showed a delayed growth (Fig. 4A), while the growth of the bo_3-expressing strain was severely impaired (Fig. 4B). Lacking *bd* oxidases, the latter strain proved to be highly sensitive to sulfide, with 6 μM NaHS causing ~25% reduced cell growth, as evaluated at 2 hours after NaHS addition (inset Fig. 4B). In contrast, no or very little effect on cell growth was observed over the same time window after addition of 200 μM NaHS to the strains expressing either *bd*-I or *bd*-II as the only terminal oxidase (Fig. 4C,D). Altogether, these data show that, unlike the bo_3 oxidase, the cytochromes *bd*-I and *bd*-II sustain *E. coli* growth in the presence of sulfide.

Discussion

Together with NO and CO, H_2S is presently considered a highly relevant signalling molecule in human (patho)physiology. It has long been recognized that many prokaryotes, including the model organism *E. coli* and numerous other members of the human gut microbiota, generate H_2S (see[6] and references therein). Bacteria can accomplish H_2S production by several pathways, including cysteine degradation by L-cysteine desulfhydrase, and dissimilatory sulfate reduction by SRB (see[6] and references therein). In a recent study, it was reported that orthologs of the mammalian H_2S-synthesizyng enzymes CBS, CSE and 3-MST are widespread in the bacterial world and contribute to H_2S generation, as demonstrated for several bacteria by genetic manipulation[23]. As an example, *E. coli* was shown to harbour an ortholog of 3-MST significantly contributing to bacterial H_2S synthesis. Notably, in the same study H_2S production was shown to enhance antibiotic resistance in all tested bacteria, thereby providing an adaptive advantage.

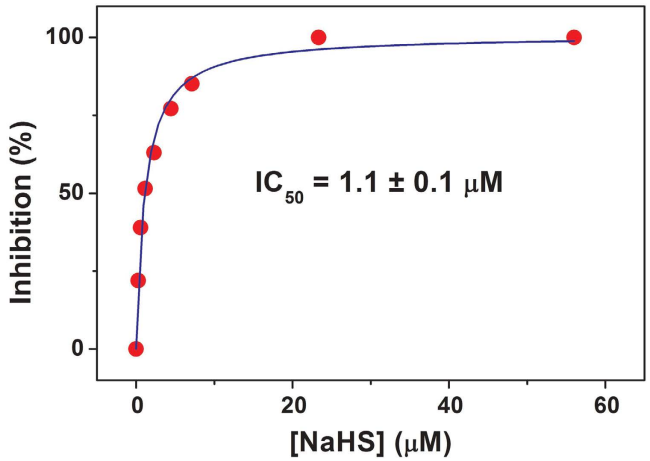

Figure 2. NaHS inhibition of isolated cytochrome *bo*$_3$. Percentage inhibition of the O_2 reductase activity of isolated cytochrome bo_3 (6 nM) measured at increasing concentration of NaHS, in the presence of the 10 mM DTT and 0.25 mM Q_1.

The presence of numerous H_2S-producing bacteria in the human gut makes this compartment particularly enriched in H_2S compared to other tissues, with the free gas reaching in the intestinal lumen concentrations as high as 40–60 μM[8–10]. Relevant to human (patho)physiology, bacteria-derived H_2S is emerging as a key regulator of several physiological functions not only in the gastrointestinal system, but also throughout the human body[1]. Moreover, it has been recently suggested that the differential susceptibility of mutualistic microbes to sulfide toxicity may contribute to shape the human gut microbiota[6], a recognized factor contributing to human health and disease. In turn, the host H_2S systemic bioavailability and metabolism have been found to be profoundly affected by the microbiota in studies on germ-free mice[24]. Altogether these observations provide evidence for interplay between H_2S and the human microbiota, with important consequences on human health.

Though currently considered a key signalling molecule, H_2S has long been known as a mere poison. Toxicity has been related to the ability of H_2S to bind heme proteins and inhibit cellular respiration targeting mtCcOX (see[3] and references therein). Indeed, H_2S is a potent ($K_i = 0.2–0.45$ μM[3,4]), non-competitive inhibitor of this respiratory enzyme, the inhibition being reversible, independent of oxygen concentration[25], but dependent on pH[26]. Sulfide inhibition of isolated mtCcOX in turnover with ascorbate and cytochrome *c* is relatively fast, occurring at an initial rate constant of $2.2 \times 10^4\ M^{-1}\ s^{-1}$, as measured at pH 7.4 [3]. The inhibited enzyme exhibits sulfide bound to ferric heme a_3[27,28], with Cu_B in the cuprous state possibly bound to a second H_2S molecule, as revealed by electron paramagnetic resonance (EPR) spectroscopy[29]. The mechanism of inhibition of mtCcOX is only partly understood, yet the reaction was suggested to involve the binding of H_2S to the enzyme in turnover at cupric or cuprous Cu_B, followed by intramolecular transfer of H_2S to ferric heme a_3, eventually blocking the reaction with O_2[3].

The well-known toxicity of H_2S on mitochondrial respiration prompted us to address whether bacterial O_2-dependent respiration can be accomplished in a H_2S-enriched environment such as the human gut, thereby providing an adaptive advantage in terms of bacterial growth. This issue was addressed in the present study working on the model organism *E. coli*, a ubiquitous member of the human gut microbiota. Namely, we investigated the effect of sulfide on the O_2 reductase activity of each of the three terminal respiratory oxidases of this bacterium (cytochromes bo_3, *bd*-I and *bd*-II), and tested the ability of these enzymes to sustain O_2 consumption and bacterial cell growth in the presence of sulfide. Using NaHS as a H_2S donor, we carried out experiments on the isolated enzymes, as well as on the wild-type and three respiratory mutant *E. coli* strains each expressing only a single terminal oxidase. NaHS is commonly used as a donor of the cell permeant H_2S, because in aqueous solution HS^- equilibrates with H_2S and S^{2-}, according to the pK_{a1} ~7.0 (H_2S/HS^-) and pK_{a2} ~19 (HS^-/S^{2-}) measured at 25 °C. At pH = 7.0–7.4, ~30–50% of HS^- is thus expected to be protonated to H_2S, with S^{2-} being present in negligible amounts.

As a new finding we report that, whereas the heme-copper bo_3 oxidase is highly sensitive to sulfide inhibition ($IC_{50} = 1.1 \pm 0.1$ μM, Figs 1 and 2), the two *bd* oxidases (*bd*-I and *bd*-II) are remarkably insensitive to sulfide (Fig. 1), as confirmed by measuring the effect of NaHS on O_2 consumption by the purified terminal oxidases (Fig. 1A) or by whole cells (Figs 1B and 3). In agreement with these finding, cell growth proved to be severely impaired by sulfide in an *E. coli* mutant strain expressing only the bo_3 oxidase (Fig. 4B), but unaffected in mutant strains expressing either *bd*-I or *bd*-II as the only terminal oxidase (Fig. 4, panel C,D). Consistently, in the wild-type strain, H_2S affected cell growth and respiration only in the early phase of the culture, when O_2 availability is expected to be still sufficiently high to favour the expression of the bo_3 oxidase, but it caused no effect in a late phase of the culture, when O_2 limitation is expected to stimulate the expression of *bd* oxidases (Fig. 4A).

Altogether, these observations led us to conclude that, at variance with the heme-copper bo_3 oxidase that is potently and reversibly inhibited by sulfide, both *E. coli bd* oxidases are sulfide-insensitive and thus able to sustain cell respiration and growth in the presence of considerably high levels of sulfide. Although the molecular basis

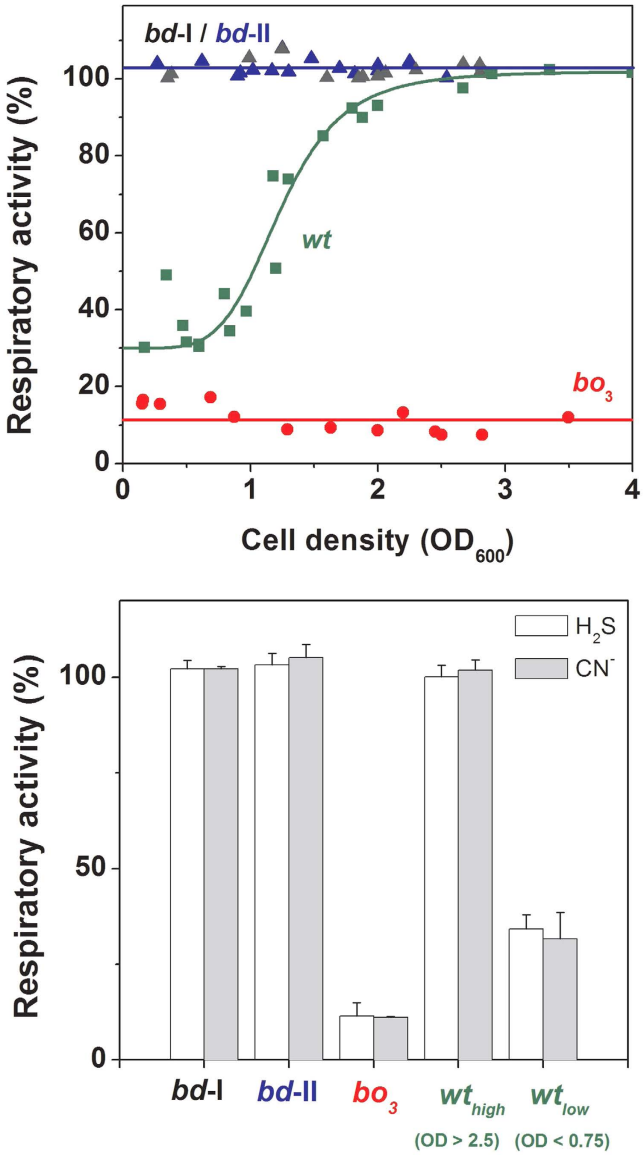

Figure 3. Effect of NaHS and cyanide on respiration of *E. coli* cells. (**Top**) Residual respiratory activity measured after the addition of 50 μM NaHS to *E. coli* cells collected at the reported cell density. (**Bottom**) Comparison of the effect of cyanide and sulfide on cell respiration: respiratory activity measured after the addition of 50 μM NaHS or 50 μM NaCN to wild-type and mutant *E. coli* cells. Data (mean ± standard deviation) refer to the control activity measured before the addition of inhibitors (taken as 100%).

for the remarkable sulfide insensitivity of the *E. coli bd* oxidases remains to be elucidated, it may originate from the lack of Cu_B, which was indeed suggested to be implicated in sulfide inhibition of mtCcOX[3]. In this regard, still possibly due to the lack of Cu_B, it is noteworthy that *bd* oxidases are not only more resistant to NO inhibition than heme-copper oxidases[30–32], but also poorly sensitive to other commonly used oxidase inhibitors, such as cyanide[12] and azide[33]. On this basis, cyanide and sulfide are expected to exert similar inhibitory effects on *E. coli* respiration, as observed in the present study (Fig. 3).

As shown here for *E. coli*, it is likely that *bd* oxidases confer sulfide resistance also to other microorganisms. The *bd* oxidases are indeed widespread in the prokaryotic world and have been identified in numerous enterobacteria[34], where expression of these oxidases is likely stimulated in the microaerobic conditions found in the human colon. In view of the novel results presented here, it will be important to test whether *bd* oxidases, by conferring sulfide resistance, play a role in shaping the human gut microbiota, thereby impacting human (patho)physiology. Furthermore, based on these data, *bd* oxidases may represent very attractive targets for the development of next-generation antimicrobials against pathogenic enterobacteria[18,20,35]. Finally, the finding that *bd* oxidases enhance bacterial resistance to sulfide, if representing a hallmark of this protein family, may pave the way to biotechnological applications aimed at increasing bacterial sulfide resistance.

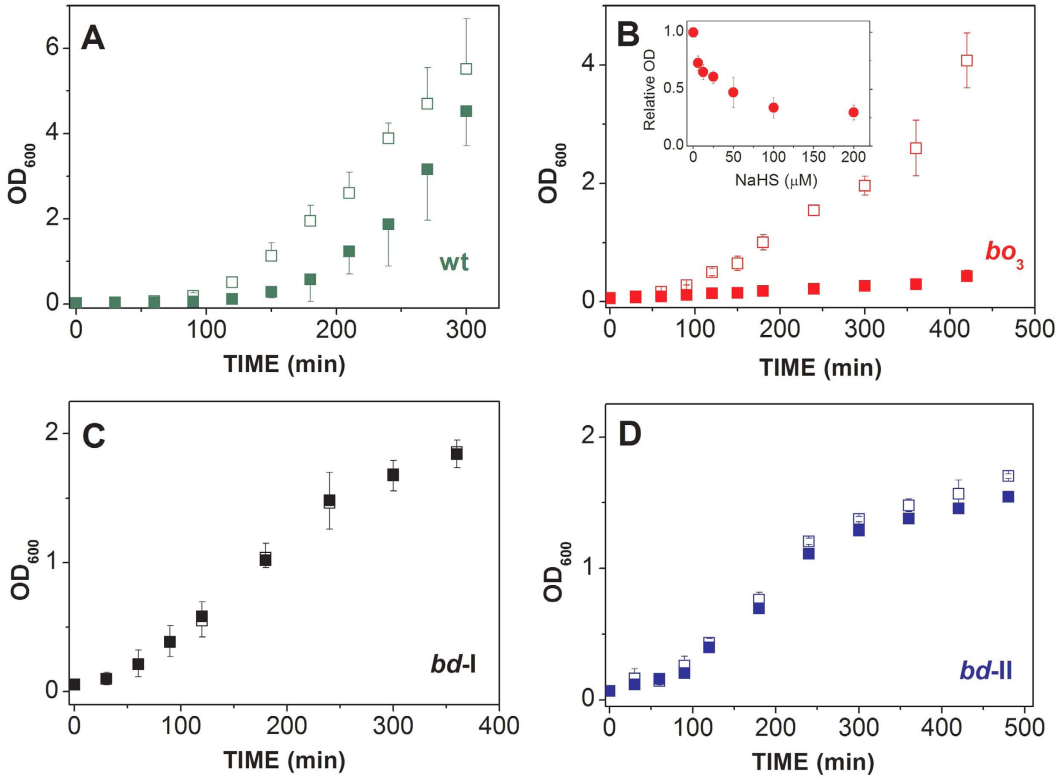

Figure 4. Effect of NaHS on *E. coli* cell growth. Cell growth of *E. coli* wild-type (**A**) and mutant strains with bo_3 (**B**), *bd-I* (**C**) or *bd-II* (**D**) as the only terminal oxidase, assayed in the presence ('closed symbols') or absence ('open symbols') of 200 μM NaHS. *Inset to panel B*: Effect of NaHS on the growth of the bo_3-only expressing mutant, as evaluated at 2 hours after addition of NaHS used at the indicated concentrations. 'Relative OD' indicates the ratio between the optical density measured at 600 nm in the presence of NaHS and the one recorded after the same period of time (2 hours) in the absence of NaHS. Data expressed as mean ± standard deviation.

Methods

Materials, bacterial strains and growth conditions. All chemicals were purchased from Sigma unless otherwise indicated. NaHS stock solutions were prepared by dissolving NaHS in degassed water or phosphate buffer saline, and the overall concentration of sulfide species ($H_2S/HS^-/S^{2-}$) in solution was determined spectrophotometrically according to[36]. All *E. coli* strains used were K-12 derivatives; MG1655 (RKP5416) was the wild type[37] from which the respiratory mutants, TBE025 (MG1655 Δ*cydB nuoB appB::kan*), TBE026 (MG1655 Δ*cydB nuoB cyoB::kan*) and TBE037 (MG1655 Δ*appB nuoB cyoB::kan*) were derived, respectively expressing cytochrome bo_3, *bd*-II and *bd*-I as the only terminal oxidase (mutants kindly given by Alex Ter Beek and Joost Teixeira de Mattos, University of Amsterdam). These strains carry the same mutant alleles as described by Bekker *et al.*[38]. *E. coli* cells were grown in 50 mL-Falcon tubes, in 5 mL Luria Bertani (LB) medium supplemented with 30 μg/mL kanamycin, at 37 °C and 200 rpm. For growth studies, cells were grown as described above in the absence or presence of NaHS (6–200 μM) added to cells at an OD_{600} of about 0.05.

Purification of terminal oxidases from *E. coli*. The cytochromes *bd*-I, *bd*-II and bo_3 were isolated from the *E. coli* strains GO105/pTK1, MB37 and GO105/pJRhisA, respectively, as previously described[39–41]. The concentration of the cytochromes *bd*-I and *bd*-II was determined from the difference absorption spectrum using $\Delta\varepsilon_{628\text{-}607} = 10.8\,mM^{-1}\,cm^{-1}$ for the dithionite-reduced *minus* 'as prepared' proteins. Cytochrome bo_3 concentration was estimated from the Soret absorption band of the oxidized enzyme using $\varepsilon_{407} = 183\,mM^{-1}\,cm^{-1}$. UV-visible absorption spectra were acquired in an Agilent Cary 60 spectrophotometer.

Purification and H_2S consumption by recombinant *O*-acetylserine sulfhydrylase from *Entamoeba histolytica*. The *O*-acetylserine sulfhydrylase-encoding gene (*Eh*OASS, Genbank XM_643199.1) was PCR-amplified from *Entamoeba histolytica* HM-1:IMSS genomic DNA using the forward primer 5′-CATATGATGGAACAAATAAGTATTAGC and the reverse primer 5′-AACGTTTTA TTCATTCAATAATGAATCAAG, containing the NdeI and HindIII restriction sites respectively. The PCR product was cloned into the Topo TA pCR2.1 vector, digested with the NdeI and HindIII restriction enzymes, and gel purified. The DNA insert was subcloned into the NdeI and HindIII restriction sites of the pET28b expression vector, yielding the pET-*Eh*OASS construct encoding N-terminally 6xHis-tagged *Eh*OASS. pET-*Eh*OASS was used to transform *E. coli* BL21 (DE3). Cells were grown at 37 °C in LB broth supplemented

with 25 mg/L kanamycin (Nzytech) until OD_{600} reached 0.4–0.5. *Eh*OASS expression was induced with 0.1 mM isopropyl-β-D-thiogalactoside addition and the cultures moved to 30 °C, 130 rpm for 4 h. Cells were harvested and the pellet resuspended in 10 mL/L culture of buffer A (50 mM potassium phosphate, 300 mM KCl, pH 7.5, 10% glycerol) containing 1 mg/mL lysozyme, 1 mM phenylmethylsulfonyl fluoride and deoxyribonuclease I. After 30-min incubation on ice, cells were disrupted by sonication, centrifuged at 8200 g (5 min, 4 °C) and imidazole was added to the supernatant to a final concentration of 10 mM. Protein purification steps were performed in an Åkta Prime (GE Healthcare) chromatography system. Affinity purification of the His-tagged protein was performed using a HisTrap FF crude 1-mL column previously equilibrated with buffer A containing 10 mM imidazole (buffer B). The cleared supernatant was loaded onto the column at 1 mL/min and the column was washed with 25 column volumes of buffer B followed by a linear gradient of 15 column volumes up to 500 mM imidazole. Pooled protein fractions were loaded onto a PD10 (GE Healthcare) desalting column for imidazole removal, equilibrated and washed with buffer A. *Eh*OASS-containing fractions were concentrated with Amicon Ultra-15 centrifugal filter units (30 kDa cut-off) and loaded onto a size-exclusion 120-ml Superdex S-200 (GE Healthcare) column, equilibrated and eluted with buffer A at 0.7 mL/min. *Eh*OASS fractions were pooled; protein purity was assessed by SDS-PAGE and protein concentration was determined by the Bradford assay. As previously reported[42], pure *Eh*OASS eluted as a dimer of ~38 kDa monomers (Figure S1).

H_2S consumption by *Eh*OASS was measured at 20 °C in 100 mM HEPES, 260 U/mL catalase, 100 μM EDTA pH 7.0, using an ISO-H2S-2 hydrogen sulfide sensor coupled to an Apollo 4000 Free Radical Analyzer (World Precision Instruments). In these assays the concentration of H_2S in solution was obtained from the nominal concentration of the NaHS added, assuming 1:1 partition between HS^- and H_2S at pH 7.0, according to the pK_a of H_2S.

O_2 consumption measurements. Oxygraphic measurements were carried out at 25 °C in 100 mM Na/phosphate pH 7.4, using a high-resolution respirometer (Oxygraph-2k, Oroboros Instruments) with a 1.5 mL chamber. The buffer was supplemented with 0.1 mM EDTA and either 0.05% *N*-lauroyl-sarcosine (cytochrome *bd*-I) or 0.02% dodecyl-β-D-maltoside (cytochrome *bd*-II and cytochrome bo_3) in the assays on isolated oxidases. The apparent IC_{50} of NaHS for the O_2-reductase activity of the isolated bo_3 oxidase was obtained by plotting the percentage inhibition of the enzyme as a function of NaHS concentration and fitting the data to the Hill equation[43], assuming a Hill coefficient $n = 1$.

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Author Contributions

E.F., V.B.B., P.S. and A.G. conceived the study and designed the experimental plan. E.F., V.B.B. and M.F. performed and analyzed the experiments with isolated oxidases and *E. coli* strains. V.B.B. produced the recombinant *bd* oxidases. H.G.C. and J.B.V. produced the recombinant *O*-acetylserine sulfhydrylase from *Entamoeba histolytica* and performed and analyzed the experiments with this enzyme. M.T.T. and R.K.P. contributed to the implementation of the experiments with the *E. coli* strains and their interpretation. A.G., V.B.B., E.F. and J.B.V. wrote the paper. All authors reviewed the results, contributed to data interpretation and critical revision of the manuscript, and approved the final version of the manuscript.

Additional Information

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