

# SCIENTIFIC REPORTS



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

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Received: 08 January 2016

Accepted: 14 March 2016

Published: 31 March 2016

Hydrogen sulfide (H<sub>2</sub>S) impairs mitochondrial respiration by potently inhibiting the heme-copper cytochrome *c* oxidase. Since many prokaryotes, including *Escherichia (E.) coli*, generate H<sub>2</sub>S and encounter high H<sub>2</sub>S levels particularly in the human gut, herein we tested whether bacteria can sustain sulfide-resistant O<sub>2</sub>-dependent respiration. *E. coli* has three respiratory oxidases, the cyanide-sensitive heme-copper *bo*<sub>3</sub> enzyme and two *bd* oxidases much less sensitive to cyanide. Working on the isolated enzymes, we found that, whereas the *bo*<sub>3</sub> oxidase is inhibited by sulfide with half-maximal inhibitory concentration  $IC_{50} = 1.1 \pm 0.1 \mu\text{M}$ , under identical experimental conditions both *bd* oxidases are insensitive to sulfide up to 58  $\mu\text{M}$ . In *E. coli* respiratory mutants, both O<sub>2</sub>-consumption and aerobic growth proved to be severely impaired by sulfide when respiration was sustained by the *bo*<sub>3</sub> oxidase alone, but unaffected by  $\leq 200 \mu\text{M}$  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<sub>2</sub>-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<sub>2</sub>S) has been recognized as an important gaseous signalling molecule, playing a major role in human (patho)physiology<sup>1</sup>. Like NO and CO, H<sub>2</sub>S 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<sub>2</sub>S can cause detrimental effects. In eukaryotes, depending on its concentration, H<sub>2</sub>S can have opposite effects on respiration (reviewed in<sup>2</sup>): at nanomolar concentrations it can sustain energy metabolism both as a substrate for the mitochondrial respiratory chain and as a vasodilator favouring O<sub>2</sub> supply, whereas at higher levels it impairs cellular respiration via direct binding to and inhibition of mitochondrial cytochrome *c* oxidase (mtCcOX) (see<sup>3</sup> and references therein). Sulfide inhibition of mtCcOX is very effective ( $K_i = 0.2\text{--}0.45 \mu\text{M}$  at pH = 7.4<sup>3,4</sup>), leading to dissipation of the mitochondrial membrane potential, consequent arrest of aerobic ATP production and eventually cell death<sup>2</sup>.

In mammalian tissues, H<sub>2</sub>S is enzymatically produced by cystathionine  $\beta$ -synthase (CBS), cystathionine  $\gamma$ -lyase (CSE) and via the combined action of 3-mercaptopyruvate sulfurtransferase (3-MST) and cysteine aminotransferase (reviewed in<sup>5</sup>). At variance from other compartments in the human body, in the intestinal lumen H<sub>2</sub>S is also generated by the gut microbiota through bacterial amino acid metabolism and *via* dissimilatory sulfate reduction by 'sulfate-reducing bacteria' (SRB)<sup>6</sup>. H<sub>2</sub>S levels in the gut are therefore high. Whereas the total sulfide pool content in the colon is around one millimolar<sup>7</sup>, the concentration of free H<sub>2</sub>S in the intestinal lumen was

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reported to be ca. 40–60  $\mu\text{M}$ , as estimated by direct measurement of the gas in the rat cecum<sup>8,9</sup> and analysis of human faecal samples<sup>10</sup>.

*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  $\text{H}_2\text{S}$ -enriched microaerobic niche, the question arises as to whether this microorganism can accomplish  $\text{O}_2$ -dependent respiration without being inhibited by  $\text{H}_2\text{S}$ . 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<sup>11,12</sup>. 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  $\text{Cu}_B$ , where  $\text{O}_2$  reduction to water takes place. On the contrary,  $bd$ -I and  $bd$ -II are cytochrome  $bd$ -type  $\text{O}_2$ -reductases phylogenetically unrelated to heme–copper oxidases<sup>12</sup>. 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  $\text{O}_2$  reduction, see<sup>12</sup> and references therein). Cytochrome  $bo_3$  predominates in *E. coli* under high aeration, whereas  $\text{O}_2$ -limiting conditions such as those found in the human gut stimulate the expression of the cytochromes  $bd$ -I and  $bd$ -II<sup>13–15</sup>. 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<sup>16</sup>. 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<sup>17–20</sup>.

In this work, we examined the effect of sulfide on the  $\text{O}_2$  reductase activity of the three terminal oxidases of *E. coli* and tested the ability of these enzymes to sustain bacterial growth and  $\text{O}_2$  consumption in the presence of sulfide.

## Results

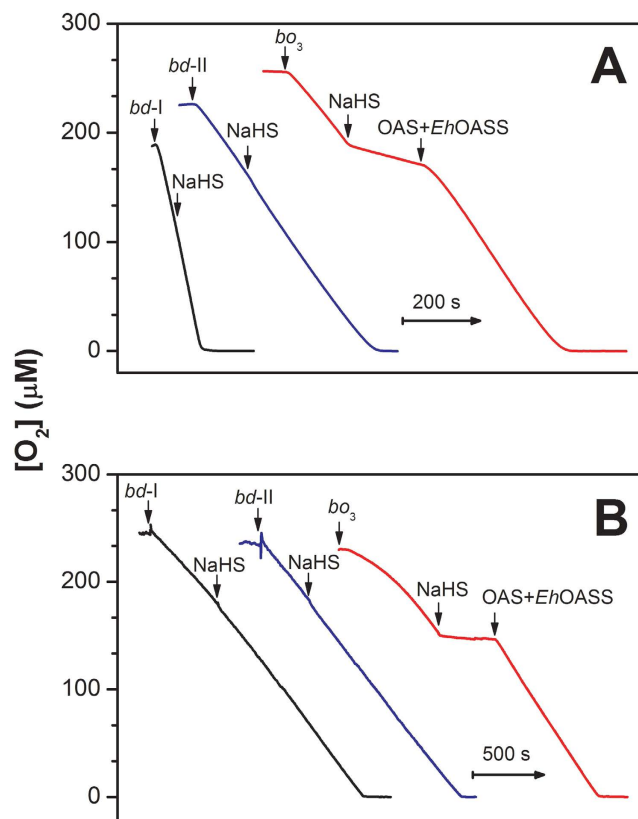
**Effect of NaHS on isolated *E. coli* terminal oxidases.** The effect of sulfide on the  $\text{O}_2$  reductase activity of the *E. coli* respiratory oxidases, cytochromes  $bo_3$ ,  $bd$ -I and  $bd$ -II, was initially investigated testing the ability of each purified oxidase to consume  $\text{O}_2$  before and after addition of the sulfide donor NaHS. In these assays,  $\text{O}_2$  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 ( $\sim 7 \mu\text{M}$ ) rapidly and effectively inhibits the  $\text{O}_2$  reductase activity of the isolated cytochrome  $bo_3$ . The enzyme is inhibited with an apparent half-maximal inhibitory concentration  $IC_{50} = 1.1 \pm 0.1 \mu\text{M}$  (Fig. 2). The inhibition of cytochrome  $bo_3$  is fully reversible. A rapid and complete recovery of the  $\text{O}_2$  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* (*EhOASS*, Fig. 1A). Sulfide consumption by *EhOASS* in the presence of OAS was assessed independently using a  $\text{H}_2\text{S}$ -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 ( $58 \mu\text{M}$ ), did not alter the  $\text{O}_2$  consumption catalyzed by the  $bd$ -I or  $bd$ -II enzyme in the presence of DTT and  $Q_1$  (Fig. 1A). No  $\text{O}_2$  consumption stimulation by the OAS/*EhOASS* 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.  $\text{O}_2$  consumption by *E. coli* cells expressing solely cytochrome  $bo_3$  was quickly and fully inhibited upon addition of  $50 \mu\text{M}$  NaHS (Fig. 1B). As observed with the isolated  $bo_3$  enzyme, the inhibition was promptly and fully restored upon sulfide depletion by the *EhOASS*/OAS system (Fig. 1B). In contrast, no inhibition was observed following the addition of  $50 \mu\text{M}$  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 ( $50 \mu\text{M}$ ) almost completely abolished  $\text{O}_2$ -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  $\text{O}_2$  availability in the medium<sup>21,22</sup>. Accordingly, when cells were assayed in an early phase of the culture ( $\text{OD}_{600} < 0.7$ ), most of respiration (65–70%) proved to be sensitive to NaHS or cyanide (both at  $50 \mu\text{M}$ , Fig. 3). In contrast, with cell growth bacterial  $\text{O}_2$ -consumption became progressively less sensitive to sulfide inhibition and, in a late phase of the culture ( $\text{OD}_{600} > 2.5$ ), 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  $\text{O}_2$ -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

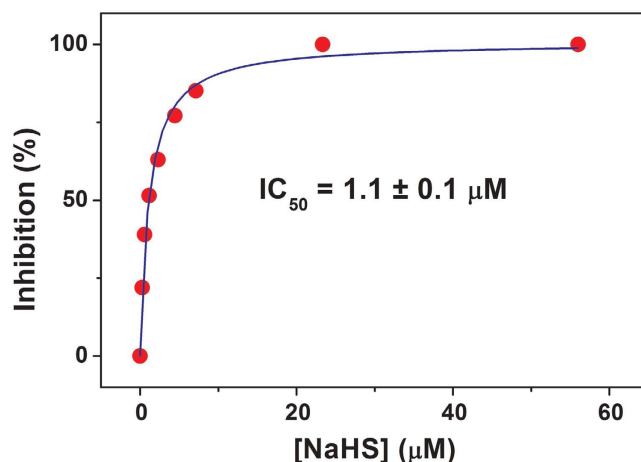


**Figure 1. Effect of NaHS on *E. coli* terminal oxidases.** (A)  $O_2$  reductase activity of the isolated cytochromes *bd-I* (20 nM), *bd-II* (2.5 nM) and *bo3* (6 nM) as measured at 25 °C in the presence of DTT (10 mM) and  $Q_1$  (0.25 mM).  $O_2$  consumption rates measured prior to NaHS addition (mean  $\pm$  standard deviation,  $n = 3$ ):  $1.62 \pm 0.07 \mu\text{M } O_2/s$  (*bd-I*);  $0.54 \pm 0.02 \mu\text{M } O_2/s$  (*bd-II*) and  $0.46 \pm 0.02 \mu\text{M } O_2/s$  (*bo3*).  $O_2$  consumption by cytochrome *bo3* is rapidly inhibited ( $\sim 85\%$ ) by 7.2  $\mu\text{M}$  NaHS, to be quickly and completely restored upon removal of sulfide from solution following the addition of 200  $\mu\text{M}$  OAS and 216 nM *EhOASS*. On the contrary, NaHS (58  $\mu\text{M}$ ) 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  $\mu\text{l}$  cells with  $OD_{600} = 1.85$ ), cytochrome *bd-II* (600  $\mu\text{l}$  cells with  $OD_{600} = 1.17$ ) or cytochrome *bo3* (200  $\mu\text{l}$  cells with  $OD_{600} = 2.45$ ), as the only terminal oxidase.  $O_2$  consumption rates measured prior to NaHS addition (mean  $\pm$  standard deviation,  $n = 3$ ):  $0.20 \pm 0.07 \mu\text{M } O_2/s$  (*bd-I*);  $0.18 \pm 0.02 \mu\text{M } O_2/s$  (*bd-II*) and  $0.19 \pm 0.02 \mu\text{M } O_2/s$  (*bo3*). When sustained solely by cytochrome *bo3*, cell respiration is rapidly inhibited by 50  $\mu\text{M}$  NaHS, to be quickly and completely restored following sulfide removal on addition of OAS (200  $\mu\text{M}$ ) and *EhOASS* (216 nM). No inhibition is observed following the addition of NaHS (50  $\mu\text{M}$ ), when respiration is sustained by the only cytochrome *bd-I* or *bd-II*.

200  $\mu\text{M}$  NaHS, the wild-type strain showed a delayed growth (Fig. 4A), while the growth of the *bo3*-expressing strain was severely impaired (Fig. 4B). Lacking *bd* oxidases, the latter strain proved to be highly sensitive to sulfide, with 6  $\mu\text{M}$  NaHS causing  $\sim 25\%$  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  $\mu\text{M}$  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 *bo3* 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<sup>6</sup> 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<sup>6</sup> and references therein). In a recent study, it was reported that orthologs of the mammalian  $H_2S$ -synthesizing 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<sup>23</sup>. 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  $Q_1$ .

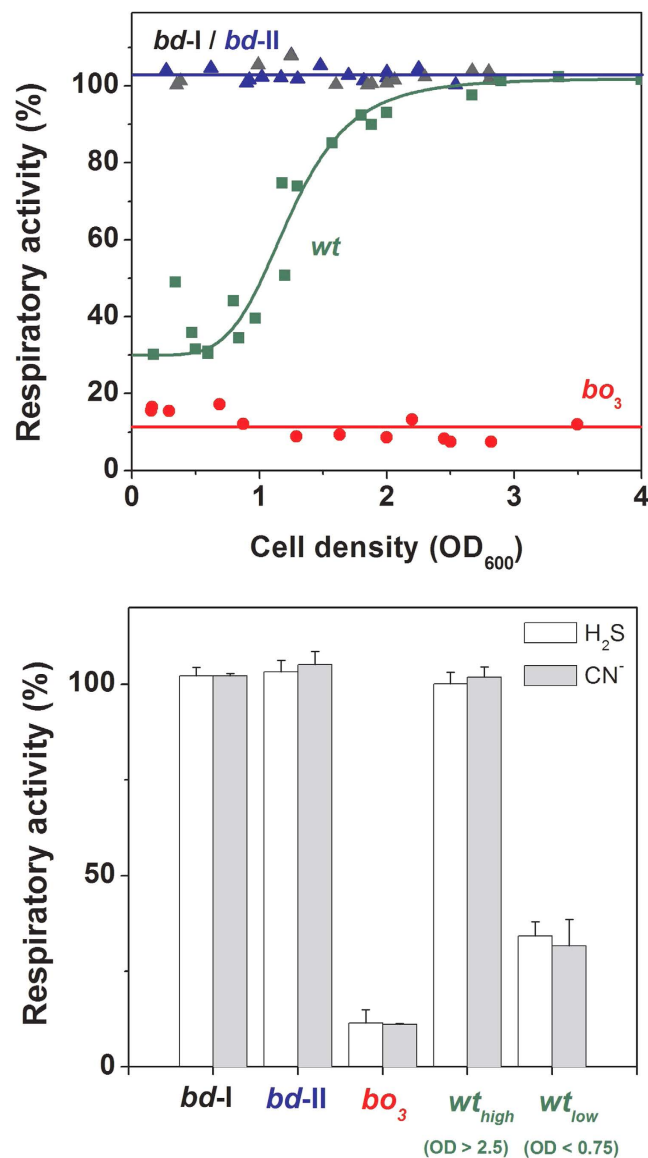
The presence of numerous  $H_2S$ -producing bacteria in the human gut makes this compartment particularly enriched in  $H_2S$  compared to other tissues, with the free gas reaching in the intestinal lumen concentrations as high as 40–60  $\mu M^{8-10}$ . Relevant to human (patho)physiology, bacteria-derived  $H_2S$  is emerging as a key regulator of several physiological functions not only in the gastrointestinal system, but also throughout the human body<sup>1</sup>. Moreover, it has been recently suggested that the differential susceptibility of mutualistic microbes to sulfide toxicity may contribute to shape the human gut microbiota<sup>6</sup>, 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<sup>24</sup>. 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<sup>3</sup> and references therein). Indeed,  $H_2S$  is a potent ( $K_i = 0.2\text{--}0.45 \mu M^{3,4}$ ), non-competitive inhibitor of this respiratory enzyme, the inhibition being reversible, independent of oxygen concentration<sup>25</sup>, but dependent on pH<sup>26</sup>. 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<sup>3</sup>. 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<sup>29</sup>. 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  $pK_{a1} \sim 7.0$  ( $H_2S/HS^-$ ) and  $pK_{a2} \sim 19$  ( $HS^-/S^{2-}$ ) 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 ( $IC_{50} = 1.1 \pm 0.1 \mu M$ , 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).

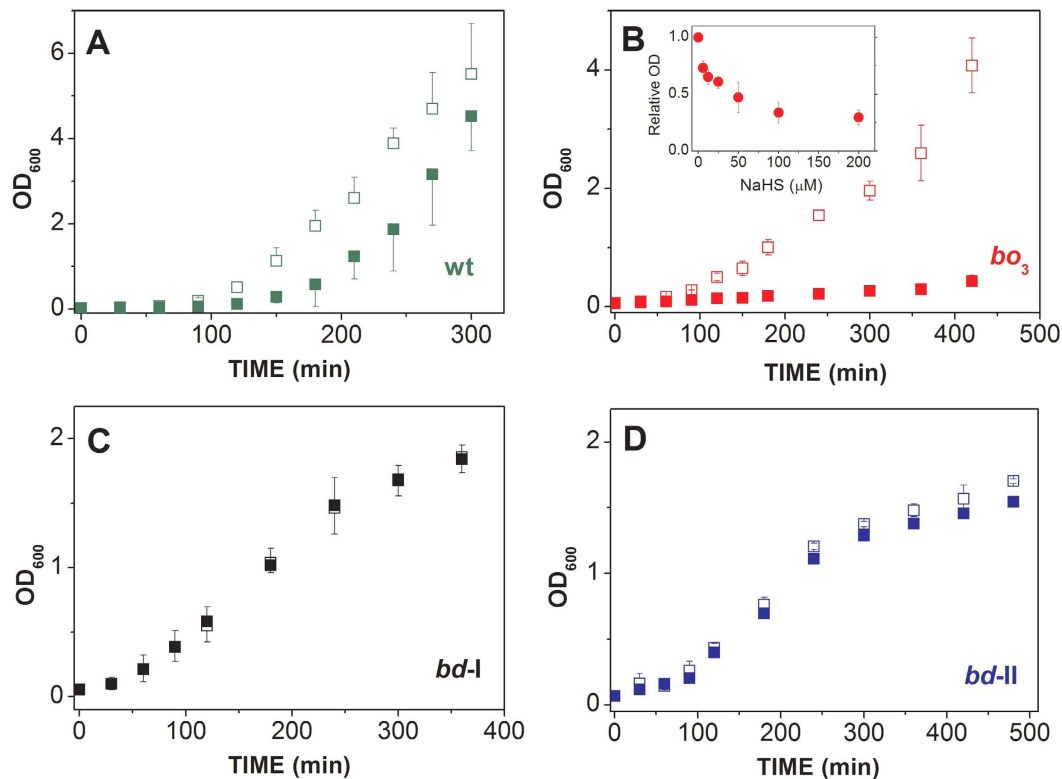
Altogether, these observations led us to conclude that, at variance with the heme-copper  $bo_3$  oxidase that is potently and reversibly inhibited by sulfide, both *E. coli*  $bd$  oxidases are sulfide-insensitive and thus able to sustain cell respiration and growth in the presence of considerably high 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  $\mu$ M 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  $\mu$ M NaHS or 50  $\mu$ M NaCN to wild-type and mutant *E. coli* cells. Data (mean  $\pm$  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<sub>B</sub>, which was indeed suggested to be implicated in sulfide inhibition of mtCcOX<sup>3</sup>. In this regard, still possibly due to the lack of Cu<sub>B</sub>, it is noteworthy that *bd* oxidases are not only more resistant to NO inhibition than heme-copper oxidases<sup>30–32</sup>, but also poorly sensitive to other commonly used oxidase inhibitors, such as cyanide<sup>12</sup> and azide<sup>33</sup>. 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<sup>34</sup>, 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<sup>18,20,35</sup>. 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*<sub>3</sub> (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*<sub>3</sub>-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<sup>36</sup>. All *E. coli* strains used were K-12 derivatives; MG1655 (RKP5416) was the wild type<sup>37</sup> from which the respiratory mutants, TBE025 (MG1655  $\Delta cydB nuoB appB::kan$ ), TBE026 (MG1655  $\Delta cydB nuoB cyoB::kan$ ) and TBE037 (MG1655  $\Delta appB nuoB cyoB::kan$ ) were derived, respectively expressing cytochrome *bo*<sub>3</sub>, *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.*<sup>38</sup>. *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<sub>600</sub> of about 0.05.

**Purification of terminal oxidases from *E. coli*.** The cytochromes *bd-I*, *bd-II* and *bo*<sub>3</sub> were isolated from the *E. coli* strains GO105/pTK1, MB37 and GO105/pJRhisA, respectively, as previously described<sup>139–41</sup>. The concentration of the cytochromes *bd-I* and *bd-II* was determined from the difference absorption spectrum using  $\Delta\epsilon_{628-607} = 10.8 \text{ mM}^{-1} \text{ cm}^{-1}$  for the dithionite-reduced *minus* ‘as prepared’ proteins. Cytochrome *bo*<sub>3</sub> concentration was estimated from the Soret absorption band of the oxidized enzyme using  $\epsilon_{407} = 183 \text{ mM}^{-1} \text{ cm}^{-1}$ . UV-visible absorption spectra were acquired in an Agilent Cary 60 spectrophotometer.

**Purification and H<sub>2</sub>S consumption by recombinant O-acetylserine sulfhydrylase from *Entamoeba histolytica*.** The O-acetylserine sulfhydrylase-encoding gene (*EhOASS*, 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′-AACGTTTATTCATTCAATAATGAATCAAG, 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-*EhOASS* construct encoding N-terminally 6xHis-tagged *EhOASS*. pET-*EhOASS* 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<sub>600</sub> reached 0.4–0.5. *EhOASS* expression was induced with 0.1 mM isopropyl- $\beta$ -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 Äkta 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. *EhOASS*-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. *EhOASS* fractions were pooled; protein purity was assessed by SDS-PAGE and protein concentration was determined by the Bradford assay. As previously reported<sup>42</sup>, pure *EhOASS* eluted as a dimer of ~38 kDa monomers (Figure S1).

H<sub>2</sub>S consumption by *EhOASS* was measured at 20 °C in 100 mM HEPES, 260 U/mL catalase, 100  $\mu$ M 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<sub>2</sub>S in solution was obtained from the nominal concentration of the NaHS added, assuming 1:1 partition between HS<sup>-</sup> and H<sub>2</sub>S at pH 7.0, according to the pK<sub>a</sub> of H<sub>2</sub>S.

**O<sub>2</sub> 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- $\beta$ -D-maltoside (cytochrome *bd*-II and cytochrome *bo*<sub>3</sub>) in the assays on isolated oxidases. The apparent IC<sub>50</sub> of NaHS for the O<sub>2</sub>-reductase activity of the isolated *bo*<sub>3</sub> 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<sup>43</sup>, assuming a Hill coefficient  $n = 1$ .

## References

- Wallace, J. L. & Wang, R. Hydrogen sulfide-based therapeutics: exploiting a unique but ubiquitous gasotransmitter. *Nat. Rev. Drug Discov.* **14**, 329–345 (2015).
- Szabo, C. *et al.* Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. *Br. J. Pharmacol.* **171**, 2099–2122 (2014).
- Nicholls, P., Marshall, D. C., Cooper, C. E. & Wilson, M. T. Sulfide inhibition of and metabolism by cytochrome *c* oxidase. *Biochem. Soc. Trans.* **41**, 1312–1316 (2013).
- Cooper, C. E. & Brown, G. C. The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: chemical mechanism and physiological significance. *J. Bioenerg. Biomembr.* **40**, 533–539 (2008).
- Kabil, O. & Banerjee, R. Enzymology of H<sub>2</sub>S biogenesis, decay and signaling. *Antioxid. Redox Signal.* **20**, 770–782 (2014).
- Carbonero, F., Benefiel, A. C., Alizadeh-Ghamsari, A. H. & Gaskins, H. R. Microbial pathways in colonic sulfur metabolism and links with health and disease. *Front. Physiol.* **3**, 448 (2012).
- Macfarlane, G. T., Gibson, G. R. & Cummings, J. H. Comparison of fermentation reactions in different regions of the human colon. *J. Appl. Bacteriol.* **72**, 57–64 (1992).
- Suarez, F., Furne, J., Springfield, J. & Levitt, M. Production and elimination of sulfur-containing gases in the rat colon. *Am. J. Physiol.* **274**, G727–G733 (1998).
- Levitt, M. D., Springfield, J., Furne, J., Koenig, T. & Suarez, F. L. Physiology of sulfide in the rat colon: use of bismuth to assess colonic sulfide production. *J. Appl. Physiol.* **92**, 1655–1660 (2002).
- Jorgensen, J. & Mortensen, P. B. Hydrogen sulfide and colonic epithelial metabolism: implications for ulcerative colitis. *Dig. Dis. Sci.* **46**, 1722–1732 (2001).
- Poole, R. K. & Cook, G. M. Redundancy of aerobic respiratory chains in bacteria? Routes, reasons and regulation. *Adv. Microb. Physiol.* **43**, 165–224 (2000).
- Borisov, V. B., Gennis, R. B., Hemp, J. & Verkhovskiy, M. I. The cytochrome *bd* respiratory oxygen reductases. *Biochim. Biophys. Acta* **1807**, 1398–1413 (2011).
- Rolfe, M. D. *et al.* Transcript profiling and inference of *Escherichia coli* K-12 ArcA activity across the range of physiologically relevant oxygen concentrations. *J. Biol. Chem.* **286**, 10147–10154 (2011).
- Ederer, M. *et al.* A mathematical model of metabolism and regulation provides a systems-level view of how *Escherichia coli* responds to oxygen. *Front. Microbiol.* **5**, 124 (2014).
- Bettenbrock, K. *et al.* Towards a systems level understanding of the oxygen response of *Escherichia coli*. *Adv. Microb. Physiol.* **64**, 65–114 (2014).
- Puustinen, A., Finel, M., Haltia, T., Gennis, R. B. & Wikström, M. Properties of the two terminal oxidases of *Escherichia coli*. *Biochemistry* **30**, 3936–3942 (1991).
- Lindqvist, A., Membrillo-Hernandez, J., Poole, R. K. & Cook, G. M. Roles of respiratory oxidases in protecting *Escherichia coli* K12 from oxidative stress. *Antonie Van Leeuwenhoek* **78**, 23–31 (2000).
- Giuffrè, A., Borisov, V. B., Arese, M., Sarti, P. & Forte, E. Cytochrome *bd* oxidase and bacterial tolerance to oxidative and nitrosative stress. *Biochim. Biophys. Acta* **1837**, 1178–1187 (2014).
- Borisov, V. B., Forte, E., Siletsky, S. A., Sarti, P. & Giuffrè, A. Cytochrome *bd* from *Escherichia coli* catalyzes peroxynitrite decomposition. *Biochim. Biophys. Acta* **1847**, 182–188 (2015).
- Borisov, V. B. *et al.* Cytochrome *bd* protects bacteria against oxidative and nitrosative stress: a potential target for next-generation antimicrobial agents. *Biochemistry-Moscow* **80**, 565–575 (2015).
- Alexeeva, S., Hellingwerf, K. & Teixeira de Mattos, M. J. Quantitative assessment of oxygen availability: Perceived aerobiosis and its effect on flux distribution in the respiratory chain of *Escherichia coli*. *J. Bacteriol.* **184**, 1402–1406 (2002).
- Cotter, P. A., Chepuri, V., Gennis, R. B. & Gunsalus, R. P. Cytochrome *o* (*cyoABCDE*) and *d* (*cydAB*) oxidase gene expression in *Escherichia coli* is regulated by oxygen, pH, and the *fnr* gene product. *J. Bacteriol.* **172**, 6333–6338 (1990).

23. Shatalin, K., Shatalina, E., Mironov, A. & Nudler, E. H<sub>2</sub>S: a universal defense against antibiotics in bacteria. *Science* **334**, 986–990 (2011).
24. Shen, X. *et al.* Microbial regulation of host hydrogen sulfide bioavailability and metabolism. *Free Radic. Biol. Med.* **60**, 195–200 (2013).
25. Petersen, L. C. The effect of inhibitors on the oxygen kinetics of cytochrome *c* oxidase. *Biochim. Biophys. Acta* **460**, 299–307 (1977).
26. Nicholls, P. & Kim, J. K. Sulphide as an inhibitor and electron donor for the cytochrome *c* oxidase system. *Can. J. Biochem.* **60**, 613–623 (1982).
27. Nicholls, P. The effect of sulphide on cytochrome *aa*<sub>3</sub>. Isosteric and allosteric shifts of the reduced *a*-peak. *Biochim. Biophys. Acta* **396**, 24–35 (1975).
28. Nicholls, P., Petersen, L. C., Miller, M. & Hansen, F. B. Ligand-induced spectral changes in cytochrome *c* oxidase and their possible significance. *Biochim. Biophys. Acta* **449**, 188–196 (1976).
29. Hill, B. C. *et al.* Interactions of sulphide and other ligands with cytochrome *c* oxidase. An electron-paramagnetic-resonance study. *Biochem. J.* **224**, 591–600 (1984).
30. Borisov, V. B. *et al.* Redox control of fast ligand dissociation from *Escherichia coli* cytochrome *bd*. *Biochem. Biophys. Res. Commun.* **355**, 97–102 (2007).
31. Mason, M. G. *et al.* Cytochrome *bd* confers nitric oxide resistance to *Escherichia coli*. *Nat. Chem. Biol.* **5**, 94–96 (2009).
32. Giuffrè, A., Borisov, V. B., Mastronicola, D., Sarti, P. & Forte, E. Cytochrome *bd* oxidase and nitric oxide: From reaction mechanisms to bacterial physiology. *FEBS Lett.* **586**, 622–629 (2012).
33. Poole, R. K., Williams, H. D., Downie, J. A. & Gibson, F. Mutations affecting the cytochrome *d*-containing oxidase complex of *Escherichia coli* K12: Identification and mapping of a fourth locus, *cydD*. *J. Gen. Microbiol.* **135**, 1865–1874 (1989).
34. Degli Esposti, M. *et al.* Molecular evolution of cytochrome *bd* oxidases across proteobacterial genomes. *Genome Biol. Evol.* **7**, 801–820 (2015).
35. Cook, G. M., Greening, C., Hards, K. & Berney, M. Energetics of pathogenic bacteria and opportunities for drug development. *Adv. Microb. Physiol.* **65**, 1–62 (2014).
36. Nashef, A. S., Osuga, D. T. & Feeney, R. E. Determination of hydrogen sulfide with 5,5'-dithiobis-(2-nitrobenzoic acid), N-ethylmaleimide, and parachloromercuribenzoate. *Anal. Biochem.* **79**, 394–405 (1977).
37. Blattner, F. R. *et al.* The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453–1462 (1997).
38. Bekker, M., de Vries, S., Ter Beek, A., Hellingwerf, K. J. & de Mattos, M. J. Respiration of *Escherichia coli* can be fully uncoupled via the nonelectrogenic terminal cytochrome *bd*-II oxidase. *J. Bacteriol.* **191**, 5510–5517 (2009).
39. Puustinen, A., Verkhovskiy, M. I., Morgan, J. E., Belevich, N. P. & Wikström, M. Reaction of the *Escherichia coli* quinol oxidase cytochrome *bo*<sub>3</sub> with dioxygen: The role of a bound ubiquinone molecule. *Proc. Natl. Acad. Sci. USA* **93**, 1545–1548 (1996).
40. Borisov, V. B. Interaction of *bd*-type quinol oxidase from *Escherichia coli* and carbon monoxide: Heme *d* binds CO with high affinity. *Biochemistry-Moscow* **73**, 14–22 (2008).
41. Borisov, V. B. *et al.* Aerobic respiratory chain of *Escherichia coli* is not allowed to work in fully uncoupled mode. *Proc. Natl. Acad. Sci. USA* **108**, 17320–17324 (2011).
42. Krishna, C. *et al.* Crystallization and preliminary crystallographic analysis of cysteine synthase from *Entamoeba histolytica*. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **63**, 512–515 (2007).
43. Goutelle, S. *et al.* The Hill equation: a review of its capabilities in pharmacological modelling. *Fundam. Clin. Pharmacol.* **22**, 633–648 (2008).

## Acknowledgements

This work was partly supported by: Ministero dell'Istruzione, dell'Università e della Ricerca of Italy (PNR-CNR Aging Program 2012–2014 to A.G. and PRIN 20107Z8XBW\_005 to P.S.); Regione Lazio of Italy (FILAS-RU-2014 – 1020); Russian Foundation for Basic Research (research projects № 14-04-00153 and 15-04-06266 to V.B.B.); Fundação para a Ciência e Tecnologia (FCT) of Portugal (Grant PTDC/SAU-MIC/111447/2009 to J.B.V.); a bilateral grant award by Consiglio Nazionale delle Ricerche of Italy (CNR) of Italy and FCT of Portugal (to A.G. and J.B.V.). iNOVA4Health - UID/Multi/04462/2013, a program financially supported by FCT/Ministério da Educação e Ciência, through national funds and co-funded by FEDER under the PT2020 Partnership Agreement is acknowledged. V.B.B. was the recipient of a short-term fellowship by CNR of Italy. We thank Prof. R.B. Gennis (Urbana, USA) for the *E. coli* strain GO105/pTK1, Dr. M. Bekker (Amsterdam, Netherlands) for the *E. coli* strain MB37 and the *E. coli* oxidase mutants, Dr. M. Verkhovskaya (Helsinki, Finland) for the purified *E. coli bo*<sub>3</sub> oxidase, and Dr. Upinder Singh (Stanford University, CA, USA) for the *Entamoeba histolytica* HM-1:IMSS genomic DNA.

## 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

**Supplementary information** accompanies this paper at <http://www.nature.com/srep>

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Forte, E. *et al.* The Terminal Oxidase Cytochrome *bd* Promotes Sulfide-resistant Bacterial Respiration and Growth. *Sci. Rep.* **6**, 23788; doi: 10.1038/srep23788 (2016).



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