Cardiolipin (CL) is a lipid that is found in the membranes of bacteria and the inner membranes of mitochondria. CL can increase the activity of integral membrane proteins, in particular components of respiratory pathways. We here report that CL activated detergent-solubilized cytochrome bd, a terminal oxidase from *Escherichia coli*. CL enhanced the oxygen consumption activity ~ twofold and decreased the apparent $K_M$ value for ubiquinol-1 as substrate from 95 µM to 35 µM. Activation by CL was also observed for cytochrome bd from two Gram-positive species, *Geobacillus thermodenitrificans* and *Corynebacterium glutamicum*, and for cytochrome bo$_3$ from *E. coli*. Taken together, CL can enhance the activity of detergent-solubilized cytochrome bd and cytochrome bo$_3$.

Cardiolipin (CL) is an anionic phospholipid that consists of two phosphatidyl groups connected by a glycerol moiety. CL is important for optimal function of various eukaryotic and prokaryotic membrane protein complexes. CL can interact with bacterial respiratory complexes from phylogenetically diverse species, such as *Mycobacterium phlei*, *Rhodobacter sphaeroides* and *Escherichia coli*. Among *E. coli* respiratory chain complexes, CL was shown to activate purified, detergent-free cytochrome bo$_3$ and was the most efficient phospholipid for activation of detergent-solubilized NADH dehydrogenase and of liposome-reconstituted nitrate reductase. Defined binding sites for CL have been determined in crystal structures of *E. coli* formate dehydrogenase, succinate dehydrogenase and nitrate reductase.

The respiratory chain in *Escherichia coli* features a heme-copper-type terminal oxidase, cytochrome bo$_3$, which transfers electrons from quinol-type substrates onto molecular oxygen. Next to this energetically efficient terminal oxidase, *E. coli* utilizes cytochrome bd as an alternative branch of the respiratory chain. Cytochrome bd oxidizes quinols, like ubiquinol or menaquinol, coupled with reduction of molecular oxygen to water. Cytochrome bd is particularly important under conditions of stress, such as O$_2$-limitation, in the presence of nitric oxide, hydrogen peroxide and hydrogen sulfide. Lack of cytochrome bd in uropathogenic *E. coli* strains led to attenuation in mouse infection models.

Cytochrome bd is present in a broad variety of Gram-positive and Gram-negative bacteria and archaea, but not in the respiratory chain of eukaryotes. Purified active cytochrome bd has been prepared from several bacterial species, including *E. coli*, *Azotobacter vinelandii*, *Corynebacterium glutamicum* and *Geobacillus thermodenitrificans*. However, to our knowledge there are no data available concerning the effect of CL on cytochrome bd activity.

In this report, we investigated the influence of CL on detergent-purified cytochrome bd. We found that CL activated the enzymatic activity of cytochrome bd from *E. coli*, *G. thermodenitrificans* and *C. glutamicum*. We then extended our experimentation and also assessed the impact of CL on the activity of purified cytochrome bo$_3$ from *E. coli*.
Results

CL enhances the activity and decreases the $K_M$ value of purified *E. coli* cytochrome *bd*. Cytochrome *bd* was purified from *E. coli* strain MB43 using streptactin affinity chromatography and β-D-dodecyl-maltoside (DDM) as detergent, as performed earlier\(^{32}\). The purity and spectroscopic properties of the isolated protein were comparable to previous results\(^{31,32}\) (data not shown). Blue-Native PAGE showed that the sample was devoid of large-scale aggregation (Suppl. Figure 1). In line with earlier data\(^{31,32,33}\), the purified enzyme showed a specific oxygen consumption activity of ~110 μmol O$_2$·mg$^{-1}$·min$^{-1}$ in buffer containing 0.025% DDM, using ubiquinol-1 as substrate. We examined the effect of CL and observed a twofold activation of the oxygen consumption activity (Fig. 1B). The activity of cytochrome *bd* in both the absence and the presence of CL was strongly suppressed by aurachin D (Fig. 1C), an inhibitor of *E. coli* cytochrome *bd*\(^{33}\).

We then investigated whether activation of cytochrome *bd* by CL is only observed at saturating substrate concentrations or if the $K_M$ value changes as well. In the absence of CL, cytochrome *bd* showed a $K_M$ value of 95 ± 16 μM for ubiquinol-1 as substrate, in line with previously published results\(^{25,26,34}\). In the presence of 10 μM
CL, the $K_M$ value decreased to 35 ± 4 μM (Fig. 1D). These results show that CL can influence enzymatic parameters of DDM-solubilized E. coli cytochrome bd.

**CL activates purified cytochrome bd from Gram-positive bacteria.** Next, we evaluated if activation by CL can also be found for cytochrome bd purified from other bacteria. Genetic classification analyses indicated that two basic types of cytochrome bd can be distinguished, based on the length of a hydrophilic loop (Q-loop) close to the substrate binding site in cytochrome bc3. Whereas E. coli cytochrome bd displays a long Q-loop, cytochrome bd from Gram-positive bacteria harbors a short version. Previously, purification of cytochrome bd from the two Gram-positive strains Geobacillus thermodenitrificans (formerly called Bacillus steatorrhodophilus) and Corynebacterium glutamicum was described. As observed above for the E. coli enzyme, purity and spectroscopic properties of these isolated proteins were comparable to previous results (data not shown) and the samples were devoid of large-scale aggregation (Suppl. Figure 1).

We examined the oxygen consumption activity of purified cytochrome bd from both strains with the same protocol as for E. coli cytochrome bd, except for using menaquinol-1 instead of ubiquinol-1 as substrate, as these Gram-positive bacteria use menaquinone as main constituent of the quinone pool. Cytochrome bd from G. thermodenitrificans showed lower oxygen consumption activity (~ 18 μmol O₂·mg⁻¹·min⁻¹ in the initial phase) as compared to the E. coli enzyme, consistent with previous data. After the initial phase of the reaction, time-dependent inactivation was observed (Fig. 2A). CL significantly increased the activity of cytochrome bd from this strain (Fig. 2A). As observed above for the E. coli enzyme, the activity of G. thermodenitrificans cytochrome bd was sensitive to inhibition by aurachin D in the presence and absence of CL (Fig. 2B).

Consistent with previous results, the oxygen consumption activity of cytochrome bd from C. glutamicum (~ 50 μmol O₂·mg⁻¹·min⁻¹) was lower than that of the E. coli enzyme, but higher than that of G. thermodenitrificans cytochrome bd. Importantly, the activity was significantly enhanced by CL (Fig. 2C). We confirmed that the observed oxygen consumption activity in the presence and absence of CL was sensitive to inhibition by aurachin D (Fig. 2D). These results reveal that activation by CL is not restricted to cytochrome bd from E. coli, but can also be found for this enzyme isolated from two Gram-positive bacteria.

**CL activates enzymatic activity of cytochrome bo₃ from E. coli.** We then extended our efforts to the second terminal oxidase found in E. coli, cytochrome bo₃. Cytochrome bo₃ is a heme-copper-type quinol oxidase and evolutionary is not related to cytochrome bd. Cytochrome bo₃ was purified from E. coli strain GO105/pRhisA using DDM as detergent without significant aggregation (Suppl. Figure 1), displaying similar spectroscopic properties as described earlier (data not shown). Like cytochrome bd, cytochrome bo₃ can accept ubiquinol-1 as electron donor and reduces molecular oxygen (Fig. 3A). In the absence of CL, cytochrome bo₃ displayed a specific oxygen consumption activity of 47 μmol O₂·mg⁻¹·min⁻¹, comparable to previously reported values. Addition of CL caused a pronounced increase in activity (Fig. 3B). Oxygen consumption by cytochrome bo₃ in the absence and in the presence of CL was highly susceptible to the inhibitor potassium cyanide (KCN) (Fig. 3C). The $K_M$ value decreased from 56 ± 13 μM in the absence of CL to 38 ± 4 μM in the presence of CL (Fig. 3D). Previously, a $K_M$ of 59 μM has been reported for cytochrome bo₃ in the presence of CL in the detergent-free state. Taken together, our results show that CL can activate both terminal oxidases in E. coli.

**Discussion**

It has been established that CL can influence the activity of various bacterial membrane proteins, including complexes of both aerobic and of anaerobic respiration. Previously, activation of purified cytochrome bo₃ by CL and activation of purified cytochrome bd by asolectin was reported. However, these experiments were carried out in detergent-free state. In the absence of detergent, membrane protein aggregation likely causes a significant decrease in activity, which subsequently is relieved by addition of lipid. In this study, we found that CL enhanced the activity of both terminal oxidases of the E. coli respiratory chain in the detergent-solubilized state. In line with our results, recently high enzymatic activity (889 e⁻·s⁻¹ ≥ 135 μmol O₂·mg⁻¹·min⁻¹) has been reported for E. coli cytochrome bd solubilized in MSP1D1/POPC-containing nano-discs, likely reflecting the importance of the lipid environment for the performance of this enzyme.

CL can be located at the outer surface of a detergent-solubilized membrane protein, enabling proper vertical positioning of the protein, or it may bind to clefts or cavities on the protein surface. CL may play a structural role, e.g. by binding at the interface between individual subunits, as previously reported for formate dehydrogenase. Alternatively, CL may enhance the interaction with the quinol substrate and/or facilitate the electron transfer reaction, as reported for nitrate reductase, where CL binds to a niche near the quinol-binding site. In the respiratory chain of Saccharomyces cerevisiae CL stabilizes the super-complex formed by the cytochrome bc₁ complex and cytochrome c oxidase, binding at the interface of the two components. In case of mitochondrial ATP synthase, CL transiently binds to conserved lysine residues in subunit c, possibly lubricating the motion of this membrane-embedded rotary machine. As found for DDM-purified cytochrome c oxidase from bovine heart mitochondria, CL can be functionally required for optimal electron transports and proton translocation. Three-dimensional structures are available for cytochrome bd from Geobacillus thermodenitrificans and from E. coli, however, the presently achieved resolution might not allow for identification of all bound lipid molecules. The decreased $K_M$ value of E. coli cytochrome bd for ubiquinol-1 measured here indicates that CL influences the substrate binding process.

In our study we investigated cytochrome bd and cytochrome bo₃ in the detergent-solubilized state and our results therefore do not clarify if CL has a similar effect on these enzymes in the native membrane. CL as high-curvature lipid is predominantly localized at the poles in rod-shaped bacteria and may thereby influence the cellular localization of membrane protein complexes, as suggested for the SecYEG translocon. Previously,
for cytochrome bd, a distribution in mobile patches in the E. coli cytoplasmic membrane has been reported. It needs to be investigated if CL can influence function, localization or dynamics of cytochrome bd or cytochrome bo, in the native plasma membrane.
Materials and methods

Chemicals. Aurachin D was synthesized as described earlier in Li et al. 2013 and was kindly provided by Dr. Jennifer Herrmann (Helmholtz Centre for Infection Research and Pharmaceutical Biotechnology, Saarbrücken). CL was purchased from Sigma (C1649, from bovine heart, > 80% polyunsaturated fatty acid content, primarily linoleic acid). All other chemicals were bought from Sigma, unless indicated otherwise.

Purification of cytochrome bd. Cytochrome bd from E. coli was purified based on Hoeser et al. 2013, with modifications as described by Goojani et al. Briefly, E. coli MB43 carrying the pET17cydABX-Strep-tag plasmid was grown in Luria–Bertani (LB) medium with 100 µg/ml Ampicillin at 37 °C overnight with shaking at 200 rpm. The bacteria were diluted to OD_{600} = 0.01 in 800 ml LB medium with 100 µg/ml Ampicillin and incubated until reaching OD_{600} = 0.4. Then IPTG (0.45 mM final conc.) was added and the bacteria were incubated

Figure 3. Activation of E. coli cytochrome bo₃ by CL. (A) Enzymatic function of cytochrome bo₃ (PDB ID: 1FFT39, figure created with BioRender). (B) The effect of CL (final conc. 10 µM) on oxygen consumption activity by cytochrome bo₃ purified from E. coli (final conc. 5 nM) was determined using a Clark-type electrode. The reaction was initiated by addition of ubiquinone-1 + DTT (arrow), the negative control contained ubiquinone-1 and DTT, but no cytochrome bo₃. Inset: dependency of activation on the CL concentration. (C) Impact of KCN (2 mM) on oxygen consumption by cytochrome bo₃ in the presence or absence of 10 µM CL. (D) Effect of CL on the K_{M} value of purified E. coli cytochrome bo₃. Curve fit was done with a simple Michaelis–Menten analysis, R² values in the absence and presence of CL were 0.974 and 0.983, respectively. All experiments were carried out at 37 °C. Average values were calculated from at least two biological replicates; error bars represent standard deviations.
again at 37 °C, 200 rpm until reaching OD_{600} ~ 2.0. Cells were sedimented by centrifugation at 6000 g for 20 min (JA-10 rotor). The pellets were washed by phosphate buffer saline, pH 7.4, and spun down at 6000 g for 20 min. Each 15 g of wet cells were re-suspended with 75 ml of MOPS solution (50 mM 3-N-morpholino-propanesulfonic acid, 100 mM NaCl and protease inhibitor (C0mplete, Roche). The cells were disrupted by passing three times though a Stanssed cell homogenizer at 1.8 kb. Unbroken cells were centrifuged at 9500 g (Ja-3050-trirotor) for 20 min. Subsequently, the supernatant was pelleted by ultracentrifugation 250,000 g (70-ti rotor) for 75 min at 4 °C. The pellet was re-suspended in MOPS solution and the protein concentration was measured using the BCA Protein Assay kit (Pierce) as described by the manufacturer. The concentration was adjusted to 10 mg/ml and incubated in MOPS solution containing 1% DDM (final conc.) at 4 °C for an hour with gentle shaking.

Un-solubilized material was sedimented by ultracentrifugation at 250,000 g at 4 °C for 15 min (70-ti rotor). The collected supernatant was applied on streptactin column at 4 °C (cold room) and the flowthrough was collected. The column was washed with washing buffer (50 mM sodium phosphate, 300 mM NaCl, protease inhibitor (C0mplete), containing 0.01% DDM, pH 8.0) to remove unspecific protein binding and the flow-through was collected again. The elution buffer (50 mM sodium phosphate, 300 mM NaCl, protease inhibitor (C0mplete EDTA free), 0.01% DDM, and 2.5 mM desthiobiotin pH 8.0) was added to the column at 4 °C to elute the protein.

Purification of cytochrome bd from Geobacillus thermodenitrificans and from Corynebacterium glutamicum.

Cytochrome bd from G. thermodenitrificans was extracted and purified from membrane fractions of G. thermodenitrificans K1041/pSTE-cbdAB recombinant cells with two consecutive column chromatography of DEAE-Toyopearl and hydroxyapatite in the presence of 0.5% (w/v) MEGA9 + 10, as described previously in Arutyunyan et al. 2012.18. Cytochrome bd from C. glutamicum was extracted and purified from membrane fractions of C. glutamicum ActuaD/pPC4-cydaBD recombinant cells with two consecutive chromatography of hydroxyapatite and then DEAE-Toyopearl in the presence of 0.05% (w/v) DDM.

Purification of E. coli cytochrome bo3. Cytochrome bo3 was extracted and purified from E. coli cytoplasmic membranes based on Rumbley et al. 1997, with modifications as described in Hardt et al. 2018.14. E. coli cytoplasmic membranes were prepared from strain GO105/pJRhisA in which cytochrome was aerobically grown to mid-log phase at 37 °C in LB medium supplemented with 500 μM CuSO4 and 100 μg ml⁻¹ carbenicillin. Cells were harvested by centrifugation at 10,000 × g for 10 min and the pellets were washed and repelleted twice with buffer A (20 mM (3-N-morpholino-propanesulfonic acid (MOPS), 100 mM NaCl and protease inhibitor (C0mplete, Roche)) and incubated at 30 °C for 30 min with gentle inversion every 5 min. The unsolubilized material was removed by ultracentrifugation (200,000 × g, 45 min, 4 °C) and the membrane pellet was resuspended in buffer B (20 mM MOPS, 30 mM NaSO4, 25% w/w sucrose, pH 7.4). The suspension was applied to the top of a 30% w/w sucrose and washed gradient and ultracentrifugation (130,000 × g, 16 h, 4 °C) with no deceleration or breaking to separate inner membrane from outer membrane. The inner membrane fraction was removed from the sucrose gradient and washed three times with buffer A by ultracentrifugation (200,000 × g, 45 min, 4 °C). Inner membranes were then resuspended in buffer A and either used immediately for purification or stored in aliquots at −80 °C until use. To extract cytochrome bo3, inner membrane fractions were diluted to 3 mg/ml protein content with solubilization buffer (20 mM Tris HCl, pH 8.0, 5 mM MgSO4, 10% glycerol, 300 mM NaCl, 1% DDM, 10 mM imidazole) and incubated at 30 °C for 30 min with gentle inversion every 5 min. The unsolubilized material was removed by ultracentrifugation (200,000 × g, 45 min, 4 °C), and the supernatant was applied to a Nickel-Sepharose High Performance (GE Healthcare) column that was previously washed with IMAC buffer containing 30 mM imidazole and 150 mM NaCl and cytochrome bo3 was eluted with IMAC buffer containing 200 mM imidazole, 150 mM NaCl, and 20% glycerol. The red cytochrome bo3 containing fractions were pooled and concentrated to 6.57 mg mL⁻¹ using an Amicon Ultra centrifugal filter devices with 100,000 Da molecular weight cutoff.

Oxygen consumption activity assay. Oxygen consumption by purified cytochrome bd and cytochrome bo3 was measured using a Clark-type electrode as previously described in Lu et al.31, with modifications as in Goojani et al.31. Briefly, the electrode was fully aerated (212 μM O₂ at 37 °C) and calibrated with sodium hydrosulfite. The purified enzymes (final conc: 2 nM for cytochrome bd from E. coli, 10 nM for cytochrome bd from G. thermodenitrificans, 2.8 nM for cytochrome bd from C. glutamicum, 5 nM for cytochrome bo3) were pre-incubated for three minutes with CL (and with inhibitors, if applicable) in a pre-warmed (37 °C) buffer containing 50 mM 3-N-morpholino-propanesulfonic acid (MOPS), 100 mM NaCl and 0.025% DDM, pH 7.5. Ubiquinone-1 (Sigma) and menaquinone-1 (Santa Cruz Biotechnology) were dissolved in absolute ethanol (20 mM stock) and the reducing agent diithiothreitol (1 M stock) in 50 mM HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid), pH 7.5. Quinone stock and DTT stock were mixed in 1:1 volume ratio and incubated for 3 min (ubiquinone-1/DTT) or 6 min (menaquinone-1/DTT) at 37 °C. The oxygen consumption reaction was initiated by adding the quinone/DTT mixture (final concentration 200 μM quinone and 10 mM DTT) to the assay mixture, respiration was measured for 3 min. The enzymatic activity was calculated from the slope in the period 30 s—60 s after starting the reaction (linear approximation).
Data availability
The original data describing rates measured in this study are compiled in a supplementary file (Suppl. Table 1). Original time courses generated during the current study are available from the corresponding author on reasonable request.

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Author contributions
A.H.A. and A.G.H. performed experiments; A.H.A., H.L., A.G.H. and H.G.G. designed experiments and/or analyzed data; D.G.G.M. and D.B. supervised and coordinated experiments; A.H.A., H.L., A.G.H. and H.G.G. designed experiments and/or analyzed data; D.G.G.M. and D.B. wrote the manuscript with contributions from all co-authors, D.B. and D.G.G.M. supervised the overall research.

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Competing interests
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Additional information

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