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# Cytochrome *o* as a Terminal Oxidase and Receptor for Aerotaxis in *Salmonella typhimurium*

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**Cytochrome *o* was the only oxidase of the electron transport system that was present in exponentially growing *Salmonella typhimurium* ST1. Identification of cytochrome *o* was made by the (CO-reduced)-minus-(reduced) difference spectra and by the photochemical action spectrum of the relief, by light, of CO-inhibited respiration. Cytochrome *o* also functioned as the receptor for chemotaxis to oxygen (aerotaxis). The concentration of oxygen that elicits the maximum response for aerotaxis (0.7 μM) was similar to the $K_m$ for respiration (0.74 μM), and both aerotaxis and respiration were blocked by 5 mM KCN.**

Aerotaxis is the migrational response of microorganisms to a gradient of oxygen (for reviews see references 26 and 27). The accumulations of bacteria around an air bubble (11) and at the edge of a cover glass (4) were among the first aspects of microbial behavior to be studied, but it was only recently that the biochemical mechanism of aerotaxis was systematically investigated. Links (Ph.D. thesis, Rijksuniversiteit, Leiden, The Netherlands, 1955) and Clayton (6) proposed that tactic responses to oxygen and other chemical attractants were triggered by a decrease in the rate of metabolism or the energy supply to the motor apparatus. Bacteria entering a hypoxic region experience decreased respiration and oxidative phosphorylation, and they were assumed to respond to the resulting fall in intracellular ATP concentration by changing swimming direction. Adler (1, 2) subsequently demonstrated that there are specific receptors for chemoeffectors and that chemotaxis is independent of metabolic utilization of the effectors. As a result of the studies by Adler and other investigators, the Links-Clayton hypothesis was largely discarded (3, 14, 29a).

It is now evident that previous concepts of chemotaxis were oversimplified. Taylor et al. (29) demonstrated a distinction between the mechanism of chemotaxis to sugars and amino acids and the mechanism of taxis to oxygen and alternative electron acceptors for the respiratory chain. The former is independent of the respiratory chain, whereas the latter appears to be mediated by respiration-induced changes in the proton motive force. Thus, for the limited class that includes aerotaxis and electron acceptor taxis, the Links-Clayton hypothesis has some validity, although two concepts in the original presentation are incorrect. The proton motive force, and not ATP, mediates the response, and the bacteria are attracted by oxygen rather than "shocked" by anoxia (16, 29).

Many of the recent studies of aerotaxis have been with *Salmonella typhimurium*, for which the concentration of oxygen that elicits half the maximum aerotactic response ($K_{0.5}$) was reported to be ca. 0.4 μM, as estimated from a dose-response curve (16). However, no cytochrome oxidase with a $K_m$ in that range has been reported for *S. typhimurium*. The only reported terminal oxidase is cytochrome *d* (9), which has a $K_m$ of 0.02 μM in *Escherichia coli* (24). In this study the $K_m$ for respiration in *S. typhimurium* was shown to be similar to the $K_{0.5}$ for aerotaxis, and cytochrome *o* was identified as the terminal oxidase in exponentially growing cells. A preliminary account of these investigations has been presented at the 72nd Annual Meeting of the American Society of Biological Chemists, June 1981, St. Louis, Mo. (B. L. Fandrich and D. J. Laszlo, Fed. Proc. **40:**1637, 1981).

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## MATERIALS AND METHODS

**Bacterial strain and growth conditions.** The strain used in this study was *S. typhimurium* ST1, which was derived in the Koshland laboratory by selecting *S. typhimurium* LT2 for improved chemotaxis (19). Aerotaxis in strain ST1 was similar to aerotaxis in strain LT2 (unpublished data). Cells were grown aerobically in Vogel and Bonner medium E (30) with glycerol (1% [vol/vol]) as the carbon source or anaerobically in the same medium supplemented with $KNO_3$ (30 mM) or potassium furmarate (30 mM) as the electron acceptor.

**Aerotaxis assay.** Aerotaxis was assayed by a method similar to that of Laszlo and Taylor (16). Bacteria were washed twice and suspended in medium E with glucose. A 2-μl drop was spread over a 6-mm diameter on a microscope slide, and the slide was inserted into a gas flow cell with nitrogen flow. After 1 min of anaerobiosis, the flow cell was ventilated with various oxygen-nitrogen mixtures. The smooth-swimming response of the bacteria to the oxygen was observed. The duration of the response was defined as the period from the beginning of the smooth response to the time when tumbling was restored in 50% of the cells.

**Kinetics of respiration.** The $K_m$ of the terminal oxidase was determined by the method of Rice and Hempfling (24). Although this method uses a Clarke-type oxygen electrode to measure oxygen uptake in a closed reaction vessel, the problem of the hysteresis of the electrode is avoided by allowing the electrode to stabilize at zero oxygen concentration and then adding minimal amounts of oxygenated medium (8). The time course of oxygen depletion was recorded on a strip chart recorder, the oxygen concentrations at 1-s intervals were tabulated, and the $K_m$ for the terminal oxidase was calculated with a Texas Instruments TI990 computer.

**Difference spectroscopy.** Cells were grown in 12-liter batches in a bench-top fermentor (model SF-116; New Brunswick Scientific Co., Inc.), washed twice, and resus-

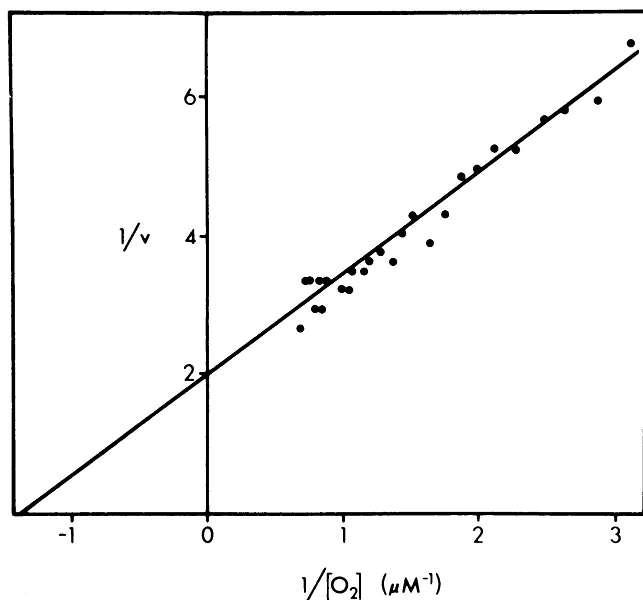


FIG. 1. Double-reciprocal plot of respiration in *S. typhimurium* ST1. The $K_m$ for respiration was determined by the procedure of Rice and Hempfling (24). A 170-ml reaction vessel (Fleaker; Corning Glass Works) was placed in a water jacket (30°C) over a magnetic stirrer and filled with strain ST1, which was suspended in medium E (30) with glucose at an optical density of 0.02 at 600 nm. The capped vessel was rendered anaerobic by respiration of the cells and flushing with nitrogen. Oxygen was added by injection of 1 to 2 ml of air-saturated buffer into the vessel. The time course of oxygen uptake was recorded by using a Clarke-type oxygen electrode (Transidyne General) and a Chemical Microsensor oxygen meter (Transidyne General). The concentration of oxygen at 2-s intervals was determined from the recording, and velocities and a Lineweaver-Burk plot of the data were obtained by using a Texas Instrument TI FS990 computer (24).

pended in buffer containing 50 mM *N*-Tris-(hydroxymethyl)methyl-2-amino-ethanesulfonic acid (pH 7.0) and 50 mM KCl. The protein concentration was determined by the biuret procedure (17), and the cell suspension was diluted to a final concentration of 10 mg of protein per ml. (Dithionite-reduced)-minus-($H_2O_2$-oxidized) and (CO-reduced)-minus-(reduced) difference spectra were obtained by using a dual-beam spectrophotometer (model DW-2a; American Instrument Co.). Anaerobic cuvettes (American Instrument Co.) with a 1-cm light path were used for studies at room temperature, and a low-temperature attachment with a cuvette with a 2-mm light path was used for spectra at 77 K. The amounts of cytochromes $b_1$, *d*, and *o* were quantified by using the extinction coefficients of Jones and Redfearn (13) and Daniel (7).

**Photochemical action spectra.** Cells were grown to mid-exponential phase, washed, and resuspended in medium E. Photochemical action spectra were determined on whole cell suspensions by using the procedure of Edwards et al. (10). This essentially consists of measuring the variation of oxygen tension of the respiring medium by using an oxygen electrode (a microelectrode made of platinum and silver) as a function of the wavelength of the probing dye laser. The monochromatic light from the dye laser (model no. 490; Coherent Radiation) pumped by an argon ion laser (model no. 96; Lexel Corp.) is tuned by using an intracavity birefringent filter. Relief of CO-inhibited respiration was measured as a rate increase from steady-state respiration in the dark. Determinations were made at room temperature.

## RESULTS

If the aerotaxis receptor is the terminal oxidase of the respiratory chain, the $K_m$ for respiration should be similar to the $K_{0.5}$, defined as the concentration of oxygen that elicits a half-maximal response in aerotaxis. The rate of respiration was determined as a function of oxygen concentration by computer analysis of the time course of oxygen consumption by *S. typhimurium* ST1 in a closed vessel (24). The $K_m$ of the terminal oxidase of the respiratory chain was 0.74 μM in exponentially growing cells (Fig. 1). This $K_m$ is similar to the $K_m$ (0.2 μM) for respiration in *E. coli* (24). For the studies of aerotaxis, a temporal assay was used in which a predetermined stepwise increase in oxygen concentration was imposed on *S. typhimurium* ST1, and the motility was observed under the microscope (16). The smooth-swimming response to the increase in oxygen concentration was timed and analyzed as a function of oxygen concentration. The $K_{0.5}$ for aerotaxis was ca. 0.7 μM (Fig. 2). Both respiration and aerotaxis were blocked by 5 mM KCN.

The studies of respiration and aerotaxis utilized *S. typhimurium* from the early exponential phase of growth (the optical density at 600 nm was 0.2 to 0.3). The (CO-reduced)-minus-(reduced) difference spectrum of similar whole cells at 23°C included a major peak at 417 nm, a trough at 432 nm, and minor peaks at 570 and 537 nm (Fig. 3). The peaks corresponded in position to those of the Soret, α, and β bands, respectively, of cytochrome *o* of *E. coli* (23). There

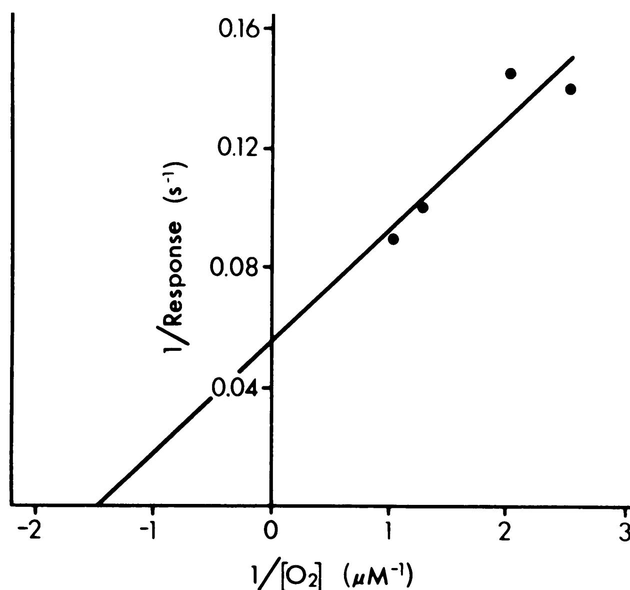


FIG. 2. Double-reciprocal plot for aerotaxis in *S. typhimurium* ST1. The bacteria were suspended in medium E (30) with glucose, and a 2-μl drop was placed in a gas flow cell, as described in the text. The flow cell was ventilated with nitrogen for 1 min, and then a predetermined oxygen-nitrogen mixture was introduced into the cell. The behavioral response of the bacteria was observed in the microscope. Timing of the response commenced when the bacteria accelerated after exposure to oxygen and ended when 50% of the bacteria resumed random swimming.

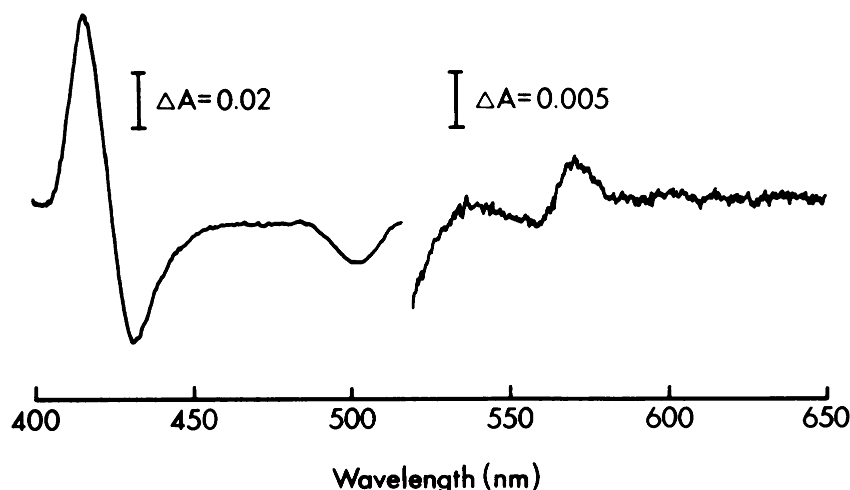


FIG. 3. (CO-reduced)-minus-(reduced) difference spectra of intact cells of *S. typhimurium* ST1. Cells were grown aerobically and harvested early in the exponential phase of growth. The contents of the sample cuvette were reduced with dithionite and bubbled with CO for 12 min. Then, dithionite was added to the contents of the reference cuvette, and difference spectra were recorded at 25°C. The final protein concentration in the cuvette was 10 mg/ml.

was no absorption peak at 625 or 590 nm, suggesting the absence of cytochromes *d* and $a_1$ (12).

The oxidase present in exponentially growing *S. typhimurium* ST1 was confirmed as cytochrome *o* by a photochemical action spectrum of the relief by light of CO-inhibited respiration (Fig. 4). A drop of cell suspension around a platinum microelectrode was held in a gas atmosphere of $CO-O_2$ in a

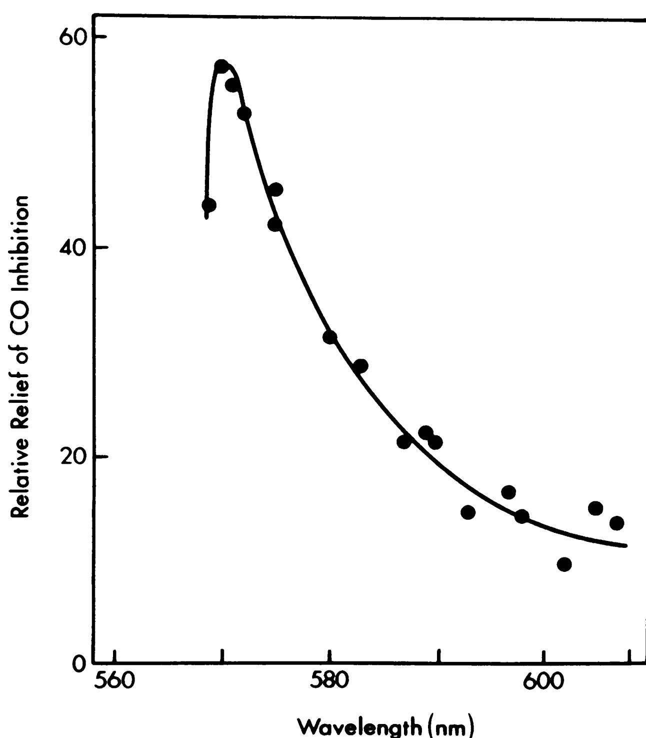


FIG. 4. Photochemical action spectrum for the relief of CO-inhibition for intact cells of *S. typhimurium* ST1 from the exponential phase of aerobic growth. The experimental procedures are described in the text. The optical density of the cell suspension at 600 nm was 0.17.

60:20 ratio. Illumination of the cells by laser light resulted in relief of CO inhibition, detected as a rapid increase in respiration rate with time (10). When the light was switched off, CO became rebound to the oxidase, and respiration was gradually inhibited, resulting in a gradual increase in oxygen tension. Maximum relief of CO inhibition was observed at 570 nm, which is the $\lambda_{max}$ of cytochrome *o* in *E. coli* (10). The wavelengths (568 to 603 nm) of laser light used in this experiment encompass the useful range of the rhodamine 6G dye. Measurements below 568 nm were not practical, but the relief of CO inhibition was consistently greater at 570 nm than at 568 or 569 nm. Cytochrome $a_1$ ($\lambda_{max}$ = 592) has been discussed as a possible oxidase in *E. coli* (10, 22) but was not present in *S. typhimurium* from early-exponential-growth phase (Fig. 4).

Aerotaxis involves a functional electron transport system in *S. typhimurium* and is observed in cells grown either aerobically or anaerobically (16). Therefore, it was of interest to determine which cytochromes, in addition to cytochrome *o*, are present in *S. typhimurium* and are potentially involved in aerotaxis. Low-temperature reduced-minus-oxidized difference spectra were obtained with whole cells of *S. typhimurium* grown under four different conditions: (i) aerobically grown, harvested in the exponential phase; (ii) aerobically grown, harvested in the stationary phase; (iii) anaerobically grown with fumarate; and (iv) anaerobically grown with nitrate. Low-temperature (CO-reduced)-minus-(reduced) spectra of the same samples were also prepared. The resulting spectra (data not shown) were similar to published spectra from *E. coli* grown under similar conditions (12). Cytochrome *o* contributes to the oxidized-minus-reduced absorption peaks at 556, 536, and 432 nm (5, 12). Additional *b*-type cytochromes ($b_{556}$ and $b_{562}$) were present in exponential-phase aerobic cells, and absorption maxima were observed at 556 and 562 nm.

The cytochrome composition changed with the growth conditions (Table 1). Cytochrome *d* (625 nm) was induced in stationary-phase aerobic cells and in cells grown anaerobically in the presence of fumarate or nitrate. A red shift in the cytochrome *d* spectrum was often observed in *S. typhimurium* grown anaerobically in the presence of fumarate or nitrate; however, the red shift was not always observed, and

TABLE 1. Cytochrome content of *S. typhimurium* ST1 grown in the presence of various electron acceptors[a]

| Electron acceptor | Cytochrome (nmol/mg of protein) | | |
|---|---|---|---|
| | $b_1$ | *d* | *o* |
| Oxygen | | | |
| Exponential phase | 0.10 | Not detected | 0.07 |
| Stationary phase | 0.18 | 0.06 | 0.05 |
| Nitrate | 0.62 | 0.02 | 0.09 |
| Fumarate | 0.12 | 0.07 | 0.03 |

[a] Cells were grown and harvested as described in the text. Reduced-minus-oxidized difference spectra were recorded at 25°C, and cytochromes were quantified by using the extinction coefficients of Jones and Redfearn (13) and Daniel (7).

the reason for the variable results was not apparent. A similar shift has been reported in *E. coli* (12). A broad absorption peak ($\lambda_{max}$ = 590 nm) attributed to cytochrome $a_1$ was observed in *S. typhimurium* from the stationary phase, or after anaerobic growth, but it was absent during aerobic exponential growth. A marked increase in absorption at 556 nm in *S. typhimurium* ST1 grown anaerobically in medium with glycerol and nitrate was presumably due to cytochrome $b_{556}$ $NO_3^-$, which is synthesized in *E. coli* under similar growth conditions (25). Cytochrome $b_{558}$ was prominent in the spectra of cells grown anaerobically with fumarate. Only minor differences in the cytochrome content of *S. typhimurium* and *E. coli* were detected, and it is likely that the sequence of carriers in the electron transport system is similar in these two species.

## DISCUSSION

Cytochrome oxidases can be identified by the ability to react with CO when they are in the ferrous state. Cytochrome *o* was the only oxidase that reacted with CO in *S. typhimurium* ST1 from the early exponential phase of growth (Fig. 3) and was the only oxidase detected in these cells by the relief by light of CO-inhibited respiration (Fig. 4). This study established that although cytochrome *o* has not been reported previously in *S. typhimurium*, it is the principal oxidase for the respiratory chain in cells grown aerobically. Aerobic cells from the stationary phase, where oxygen is growth limiting, and cells grown anaerobically contained an additional oxidase, cytochrome *d*, and cytochrome $a_1$, which may be a component of the cytochrome *d* complex (20). Cytochrome *d* has a high affinity ($K_m$ = 0.024 μM) for oxygen in *E. coli* and apparently scavenges oxygen under conditions of limited availability (12, 24). Cytochrome $a_1$ binds CO but has not been established as an independent oxidase in *E. coli* (10, 22). The synthesis of cytochromes $a_1$ and *d* are regulated coordinately.

We repeated the low-temperature spectra of *E. coli* under conditions similar to those used in obtaining the spectra for *S. typhimurium* and found no major difference between the spectra (unpublished data). The similarity of respiratory-chain components in *S. typhimurium* and *E. coli* is not surprising in view of the close genetic relationship between the two species.

The $K_{0.5}$ for aerotaxis (0.7 μM) indicates that the aerotaxis receptor is a cytochrome. No other class of proteins is known to have a $K_m$ for oxygen in the range of $10^{-6}$ to $10^{-8}$ M. The $K_{0.5}$ for aerotaxis and the $K_m$ for respiration were similar, and aerotaxis and respiration were both inhibited by KCN. These results, together with the evidence for a single oxidase in cells from the early exponential phase of growth, support the hypothesis that the aerotaxis receptor is cytochrome *o*, the terminal oxidase of the respiratory chain. Cytochrome *o* has as its prosthetic group protoporphyrin IX instead of the "green heme" of cytochrome $a_3$. It also differs somewhat from cytochrome $a_3$ in its reaction mechanism. Cytochrome *o* from *Vitreoscilla* spp. can react with oxygen or $H_2O_2$ and form a stable intermediate with $H_2O_2$ at room temperature (18). Other evidence that supports the assignment of cytochrome *o* as the aerotaxis receptor includes the following. Nitrate and fumarate are competitive with oxygen in aerotaxis in *S. typhimurium* (16). 2-Heptyl-4-hydroxyquinoline-*N*-oxide is a noncompetitive inhibitor of both respiration and aerotaxis in *Bacillus cereus*, in which cytochrome $a.a_3$ is the oxidase and receptor for aerotaxis (D. J. Laszlo, M. Niwano, W. W. Goral, and B. L. Taylor, J. Bacteriol., in press). Preliminary experiments to determine whether cytochrome *d* is also a receptor for aerotaxis were inconclusive, and definitive experiments will require adaptation of the methodology for aerotaxis to accommodate studies at very low oxygen concentrations.

Receptors for chemotaxis to sugars and amino acids are either transducing proteins (methyl-accepting chemotaxis proteins) or proteins that can interact directly with a transducing protein (15, 28). Cytochrome *o* appears not to be a receptor for aerotaxis in the usual meaning of a receptor. Recent studies that will be reported elsewhere (J. Shioi and B. L. Taylor, J. Biol. Chem., in press) indicate that the flow of electrons through the electron transport system and the consequent change in proton motive force are the signal for aerotaxis. In this respect, and in the mechanism of adaptation (21), the pathways for aerotaxis and chemotaxis are diverse.

## ACKNOWLEDGMENTS

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