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Energetic Efficiency of *Escherichia coli*: Effects of Mutations in Components of the Aerobic Respiratory Chain

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The aerobic respiratory chain of *Escherichia coli* can function with either of two different membrane-bound NADH dehydrogenases (NDH-1 and NDH-2) and with either of two ubiquinol oxidases (*bd*-type and *bo*-type). The amounts of each of these enzymes present in the *E. coli* membrane depend on growth conditions in general and particularly on the dissolved oxygen concentration. Previous in vitro studies have established that NDH-1 and NDH-2 differ in the extent to which they are coupled to the generation of an energy-conserving proton motive force. The same is true for the two ubiquinol oxidases. Hence, the bioenergetic efficiency of the aerobic respiratory chain must depend on the electron flux through each of the specific enzyme components which are being utilized. In this work, the specific rates of oxygen consumption for cells growing under glucose-limited conditions are reported for a series of isogenic strains in which one or more respiratory components are genetically eliminated. The results are compatible with the proton translocation values of the various components reported from in vitro measurements. The data show that (i) the *bd*-type oxidase is less efficient than is the *bo*-type oxidase, but the former is still a coupling site in the respiratory chain; and (ii) under the conditions employed, the wild-type strain uses both the NDH-1 and NDH-2 NADH dehydrogenases to a significant degree, but most of the electron flux is directed through the *bo*-type oxidase.

An assessment of the energetic efficiency of the respiratory chain of bacteria is of great importance from both fundamental and practical points of view. This efficiency cannot be assessed directly because, in contrast to mitochondria, ADP and ATP are not taken up or excreted, and intact organisms do not show respiratory control. Hence, indirect methods have been used, and one of the most promising is the determination of growth yields in chemostat cultures. Experiments by Bauchop and Elsden (3) provided evidence that the energy costs of biomass synthesis are similar for different bacterial species: about 10.5 g of biomass per mol of ATP. On the other hand, calculations by Stouthamer (32–34) showed that in theory, the energy costs for the synthesis of bacterial biomass should be substantially lower, i.e., 25 to 28 g/mol of ATP. Therefore, although there is uncertainty about the exact amount of energy that is needed for biomass synthesis, it is nevertheless commonly accepted that this amount is approximately constant for different bacterial species. Pathways of synthesizing macromolecules do not vary among organisms; therefore, differences in the growth yields of organisms grown under identical conditions will be a reflection of the efficiency of the energy-generating systems of the respective organisms.

The aerobic respiratory chain of *Escherichia coli* consists of (i) a set of membrane-bound dehydrogenases that feed electrons into the quinone pool in the cytoplasmic membrane and (ii) two ubiquinol oxidases that directly oxidize ubiquinol and reduce molecular oxygen to water (1, 2, 17). There is no *c*-type cytochrome, cytochrome *c* reductase, or cytochrome *c* oxidase in aerobically grown *E. coli*. Studies with *E. coli* spheroplasts or with membrane vesicles have shown that two electron transfer reactions are coupled to the generation of a proton motive force: NADH→ubiquinone (22) and ubiquinol→O_2 (27). However, *E. coli* contains two different enzymes that catalyze each of these reactions, and these enzymes differ in the degree to which they couple the electron transfer reaction to proton translocation across the membrane (Fig. 1).

The two NADH dehydrogenases that are components of the *E. coli* respiratory chain are designated NDH-1 and NDH-2 (38). NDH-1 (or NADH dhI) is the homolog of the eukaryotic mitochondrial complex I (23, 37). This multisubunit enzyme has not been purified in an intact form (13), but studies with membrane vesicles have shown that it is coupled to the generation of a proton motive force (22). The H^+/e^- ratio is not known, but by analogy with the bovine counterpart, a reasonable guess would be $2H^+/e^-$ (16). The *nuo* genetic locus encoding the subunits of the enzyme has been mapped elsewhere (4) and recently cloned (37).

NDH-2 (or NADH dhII) is a single-subunit enzyme that also catalyzes NADH:ubiquinol oxidoreductase activity but does not directly couple this reaction to any transmembrane charge movement (i.e., $H^+/e^- = 0$) (22). NDH-2 has been isolated and characterized previously (5, 19), and the *ndh* gene encoding this enzyme has been cloned and sequenced elsewhere (39, 40). The *ndh* gene has been shown to be repressed by the *fnr* gene product in such a way that expression is optimal under conditions of high oxygen concentration in the medium (29, 31). The regulation of the *nuo* operon has not yet been investigated.

The two terminal oxidases of *E. coli* also differ in their H^+/e^- translocation ratios (27). The *bd*-type oxidase, which predominates under conditions of low aeration, has an H^+/e^- of 1 (24, 27). This has been determined for the reconstituted purified protein (24) and also in situ *E. coli* with spheroplasts (27). The subunits of *bd*-type oxidase are encoded by the *cyd* operon, which has been sequenced else-

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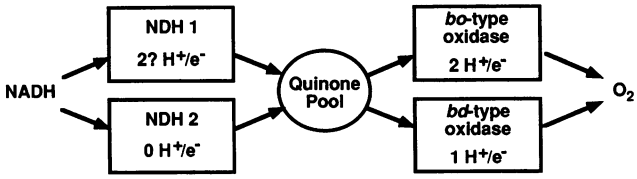

FIG. 1. A schematic representation of several components of the aerobic respiratory chain of *E. coli*. The electron flux is partitioned between the two NADH dehydrogenases and between the two quinol oxidases. The proton translocation values (per electron) of each component are indicated.

where (12) and shown to be regulated in an oxygen-dependent manner by the *fnr* and *arc* gene products (7, 10, 18). Expression appears to be optimal under microaerophilic growth conditions (10).

The *bo*-type oxidase predominates when *E. coli* is grown at high oxygen tension (2, 20, 28). Studies of this enzyme in situ with spheroplasts have clearly shown that this enzyme has an H^+/e^- of 2 (27). Studies with the reconstituted purified oxidase (21) yielded a smaller H^+/e^- of 1, probably because of a lack of a uniform orientation of the oxidase in the reconstituted proteoliposomes. The subunits of the *bo*-type oxidase are encoded by the *cyo* operon, which has been sequenced elsewhere (6) and also shown to be regulated in an oxygen-dependent manner by both the *fnr* and *arc* gene products (7, 18).

These studies make it very clear that the exact composition of the aerobic respiratory chain of *E. coli* must depend strongly on the growth conditions. Furthermore, the degree to which the respiratory chain is coupled, e.g., the H^+/e^- ratio or ATP/O ratio, will be dependent on the partitioning of the electron flux through the two NADH dehydrogenases (NDH-1 and NDH-2) and through the two oxidases (*bo*-type and *bd*-type). The partitioning of the electron flux will, in turn, depend not only on the amount of each enzyme which is present in the *E. coli* membrane but also on the effective kinetic parameters (K_m and V_{max} values).

In this work, the consequences for cell growth yields due to specific genetic deletions of respiratory components are reported. The growth yields for a set of isogenic strains were determined for cells grown in glucose-limited continuous culture. The enhanced bioenergetic efficiency of the *bo*-type oxidase ($2H^+/e^-$) compared with the *bd*-type oxidase ($1H^+/e^-$) is observed most clearly in strains that lack NDH-2 (*ndh*).

MATERIALS AND METHODS

Strains. The strains used in this study are listed in Table 1. Strains GR70N and GO103 have been previously described (11, 25). Strain GO104 was constructed by P1 transduction (30) of Δ*cyo*::kan from strain GV102 (25) into strain GR70N. The absence of the *bo*-type oxidase from strain GO104 was confirmed by spectroscopic analysis of membranes from this strain.

TABLE 1. Strains used in this work

Strain	Phenotype	Genotype	Reference or source
GR70N		F⁻ *thi rpsL gal*	11
GO103	Cyd⁻	GR70N; *zbg-2200*::kan Δ(*cydAB'*)*455*	25
GO104	Cyo⁻	GR70N; Δ(*cyoABCDE*)*456*::kan	This work
MWC215	Ndh⁻	GR70N; *ndh*::Cm^r	4
MWC217	Cyo⁻ Ndh⁻	GO104; *ndh*::Cm^r	4

The *ndh*::Cm^r mutation was constructed by in vitro insertion of a gene cartridge encoding chloramphenicol resistance from plasmid pHP45ΩCm (9) into the plasmid-encoded *ndh* gene (40). The *ndh*::Cm^r mutation was introduced into the *E. coli* chromosome via genomic replacement by methods outlined in Oden et al. (25). The *ndh*::Cm^r mutation was then transferred into strain GR70N by P1 transduction (30) to create the strain MWC215. P1 transduction was also used to transfer *ndh*::Cm^r into strain GO104 to make strain MWC217. A detailed description of the construction and characterization of the in vitro insertion in the *ndh* gene is presented elsewhere (4).

Culture methods and growth conditions. The different strains were grown in a 500-ml Modular Fermenter 500 Series II (LH Engineering Co. Ltd, Stoke Poges, Buckinghamshire, England). The glucose-limited medium as specified by Evans et al. (8) was modified as follows: glucose input was 5 g/liter (instead of 10 g/liter) and nitrilotriacetic acid (2 mM) was used instead of citrate as the chelating agent. In addition, thiamine was added at a final concentration of 15 mg/liter. The purity of the culture was checked regularly (every 2 to 4 days) by streaking a sample from the chemostat onto nutrient agar plates and onto nutrient agar plates containing kanamycin (50 μg/ml), tetracycline (12.5 μg/ml), or chloramphenicol (25 μg/ml). In addition, API 20E strips (API Systems, Marcy-l'Etoile, France) were used to check the taxonomic statues of the strains.

Analyses. The dry weights of the cultures were measured by the procedure described by Herbert et al. (15). The presence of glucose and possible extracellular products of glucose metabolism, such as acetate, was determined by high-performance liquid chromatography (HPLC) (LKB, Bromma, Sweden) with an Aminex HPX 87H Organic Acids Column (Bio-Rad, Richmond, Calif.) with 5 mM H_2SO_4 as eluent operated at a temperature of 55°C, by using a 2142 Refractive Index Detector (LKB) and an SP 4270 Integrator (Spectra Physics, San Jose, Calif.). The amounts of oxygen consumed and carbon dioxide produced by the culture were determined by passing the gas from the fermenter through an oxygen analyzer (type OA 272; Taylor Servomex, Crowborough, Sussex, England) and a carbon dioxide analyzer (IR Gas Analyzer PA404; Servomex).

Statistical analyses were carried out with the Lotus 123 computer program (release 2.01, Lotus Development Corporation, Cambridge, Mass.).

RESULTS

Each strain of *E. coli* was grown in a glucose-limited chemostat culture at different dilution rates. When the culture was in a steady state, as judged by the constancy of the dry weight of the culture and the lack of detectable glucose levels in the culture fluids, the gas from the fermenter was analyzed for its oxygen and carbon dioxide contents. Subsequently, a sample was withdrawn for a dry-weight measurement. The supernatant of this sample was used for product analysis. This was repeated for several (usually 3) days; then, the dilution rate was changed to a different setting.

The specific rates of oxygen consumption were calculated, and these are plotted in Fig. 2 and 3. Table 2 shows the

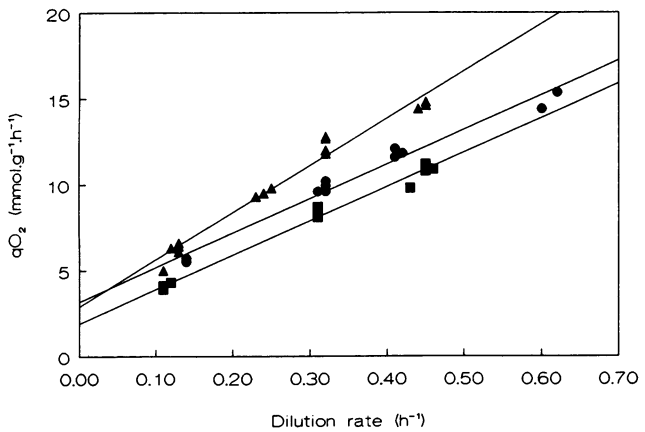

FIG. 2. Specific rates of oxygen consumption (qO_2) of the following strains of *E. coli* growing in aerobic, glucose-limited chemostat culture: GR70N (wild type [●]), MWC215 (*ndh* [■], and MWC217 (*ndh cyo* [▲]). The lines drawn are a best linear fit to the data.

relevant parameters obtained by a linear regression analysis of the data. Under all culture conditions, the carbon balances were between 90 and 100%, and no carbon-containing compounds were detectable by HPLC analysis for any of the samples of the extracellular fluids. This indicates that, in agreement with previous observations with other wild-type strains of *E. coli* (36), new cell material and carbon dioxide were the sole products of glucose metabolism.

It is clear that strains with the *bo*-type oxidase exhibit a lower specific rate of oxygen consumption than those with the *bd*-type oxidase. A further, quantitative analysis of the data is possible by calculating the ratios of the specific rates of oxygen consumption of the different strains growing at the same rate. Since it is very difficult to perform different chemostat experiments at exactly equal dilution rates, it is simpler to determine the ratios of functions describing the best linear fits to the data shown in Fig. 2 and 3. For example, the ratios of the specific rates of oxygen consumption of strains GO103 (*cyd*) and GO104 (*cyo*) and of strains MWC215 (*ndh*) and MWC217 (*ndh cyo*) were determined for

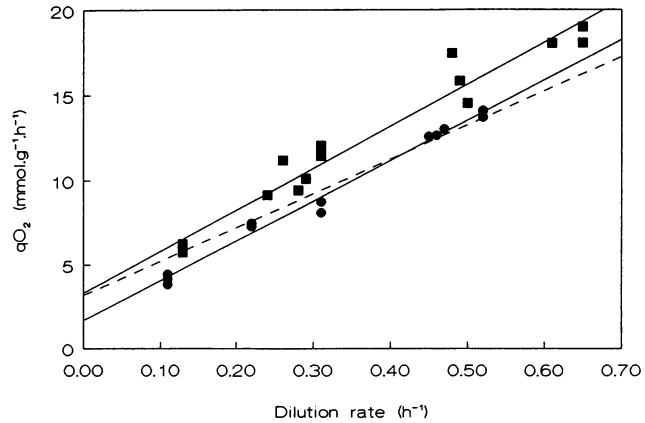

FIG. 3. Specific rates of oxygen consumption (qO_2) of *E. coli* GO103 (*cyd* [●]) and GO104 (*cyo* [■]) growing in aerobic, glucose-limited chemostat culture. The lines drawn are the best linear fit to the data. The dashed line is the best linear fit for the wild-type strain (from Fig. 2).

TABLE 2. Parameters of the lines of regression representing the best linear fits for the dependence of the specific rate of oxygen consumption on the dilution rate of different strains of *E. coli*[a]

Strain	Relevant genotype	Slope	Intercept
Wild type		20.10 ± 0.91	3.20 ± 0.49
GO103	*cyd*	23.68 ± 0.69	1.71 ± 0.40
GO104	*cyo*	24.65 ± 1.36	3.35 ± 0.99
MWC215	*ndh*	20.11 ± 0.84	1.87 ± 0.40
MWC217	*ndh cyo*	27.54 ± 1.29	2.91 ± 0.59

[a] Strains were grown in aerobic, glucose-limited chemostat culture. Data are from Fig. 1 and 2. The slope is the incremental increase in O_2 required per gram of biomass as the growth rate is increased (millimoles of O_2 per gram of biomass). The intercept is the extrapolated specific oxygen consumption rate at zero growth rate.

$0.1 \leq$ dilution rate $\leq 0.7\ h^{-1}$. The results are shown in Fig. 4. Strains which are constrained to use the *bd*-type oxidase have increased specific oxygen consumption rates by a factor of 1.45 to 1.13 (strain GO104 compared with GO103) and 1.46 to 1.39 (strain MWC217 compared with MWC215).

DISCUSSION

The goal of this work is to determine the consequences of specific respiratory defects on the growth of *E. coli*. The proton translocation values have been determined by in vitro methods (22, 27) for several enzymes: NDH-2 ($0H^+/e^-$), the *bo*-type oxidase ($2H^+/e^-$), and the *bd*-type oxidase ($1H^+/e^-$). The use of continuous culture techniques allows one to compare the energetic efficiencies of the respiratory chains of strains that lack one or more of these components. Qualitatively, the data confirm the following expectations based on the in vitro proton translocation measurements: (i) the elimination of the uncoupled NDH-2 results in increased energetic efficiency; (ii) strains that utilize the *bd*-type oxi-

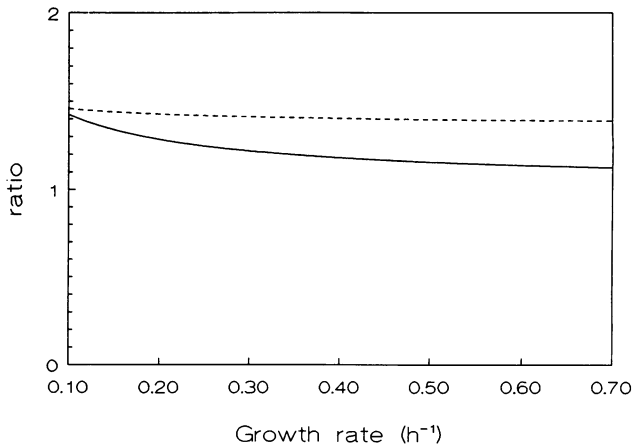

FIG. 4. Ratios of the specific rates of oxygen consumption as functions of the dilution rates of aerobic, glucose-limited chemostat cultures for the following selected pairs of strains: GO104 to GO103 (——) and MWC217 to MWC215 (- - - -). The values used to compute the ratios are from the best linear fits to the data shown in Fig. 2 and 3. In each comparison, the specific rate of oxygen consumption is higher in the strain which lacks the *bo*-type oxidase (*cyo*). Note that dilution rates of less than 0.1 are outside the range of measurements and that the data shown are from linear extrapolations.

dase have a less-efficient respiratory chain than those using the *bo*-type oxidase. A more-quantitative analysis is described below.

Background and expectations. The oxygen incorporated into cell biomass is all derived from glucose and water. Hence, the rate of consumption of O_2 is a measure of the energy required for biomass production (i.e., ATP generation) and not for oxygen assimilation. It is reasonable to assume that the same amount of energy should be needed by each different strain for the synthesis of new cell mass and for other energy-consuming processes when the strains are grown at the same rate. If this is correct, the comparison of the specific rates of oxygen consumption of various strains should be a measure of the energetic efficiency of their respiratory chains as they function in vivo. For each strain, the specific oxygen consumption rate (millimoles of O_2 consumed per hour per gram of biomass) is reported as a function of the dilution rate. A high dilution rate corresponds to a high rate of growth in the continuous culture. The oxygen concentration in the culture is essentially saturating, whereas the glucose concentration is very low, since glucose-limiting conditions were used for these experiments. The high oxygen concentration ensures that the quinone pool will consist entirely of ubiquinone and not menaquinone.

The data show that for all strains there is a linear relationship between the specific rate of oxygen consumption and the dilution rate. The specific rates of both oxygen and glucose consumption of the wild-type strain (GR70N) are in good agreement with those previously observed for a different strain of *E. coli* (36) but in sharp contrast with those observed by Hempfling and Mainzer (14; however, see reference 35).

In principle, by directing the electron flux through specific respiratory components, the energetic efficiency of the *E. coli* respiratory chain can be varied between $4H^+/e^-$ (with NDH-1 and the *bo*-type oxidase) and $1H^+/e^-$ (with NDH-2 and the *bd*-type oxidase). Since the wild-type strain contains both NADH dehydrogenases and both terminal oxidases, the value for the H^+/e^- ratio must fall between these two extremes and will vary with growth conditions. The effect on the cell growth yield due to changing the H^+/e^- ratio can be computed by considering the ATP equivalents generated per mole of glucose. The oxidation of glucose requires 6 equivalents of O_2 and results in 2 ATP, 2 GTP, 10 NAD(P)H and 2 F_P molecules, where 2 F_P indicates reducing equivalents that are directed through the oxidase site of the respiratory chain but not the NADH dehydrogenase site. Hence, per glucose there are 4 ATP (GTP) equivalents formed by substrate level phosphorylation, 20 electrons processed through the NADH dehydrogenase site, and 24 electrons which pass through the oxidase site of the respiratory chain. For example, if NDH-1 ($2H^+/e^-$) and the *bo*-type oxidase ($2H^+/e^-$) are used, one can compute a yield of 33 ATP equivalents per glucose if it is assumed that 3 protons per ATP molecule are required by the ATP synthase. If the *bd*-type oxidase ($1H^+/e^-$) instead of the *bo*-type oxidase ($2H^+/e^-$) were used, the ATP equivalents per glucose would drop to 25, and this would require an increase in the specific oxygen consumption of 33/25, or 1.32-fold, to maintain the same rate of ATP generation. Although this calculation yields a reasonable estimate of the expected change in the specific rate of oxygen consumption due to switching the oxidases, the quantitative aspects are dubious, since the exact values of the H^+/e^- and H^+/e^- ratios are not known. Traditionally, microbial physiologists have assumed a value of 26 ATP equivalents per glucose for a bacterial respiratory chain with two coupling sites (34), considerably less than the value of 33 estimated above. However, the ATP/glucose ratio that one uses makes little difference in the estimated effects of changing the proton translocation efficiency of the respiratory chain.

The analysis used in this work directly compares the specific oxygen consumption rates for different strains, as shown in Fig. 4. Alternatively, the slope and intercept values from Table 2 can be utilized. These values show the dependence of the specific oxygen consumption rate on the growth rate. The slope is the incremental increase in the rate of oxygen consumption per gram of biomass, and the intercept is a measure of the oxygen consumption rate extrapolated to a growth rate of zero. Both the slope and intercept parameters are affected by the respiratory mutations, but the metabolic processes that contribute to each of these parameters are not known. A simple comparison of the slopes does not take into account the differences in the maintenance energy requirements of the strains (26). For these reasons, the total specific oxygen consumption rates are used, as in Fig. 4, in comparing isogenic strains.

The effect of the genetic elimination of NDH-2. Figure 2 compares the wild-type strain (GR70N) with an isogenic strain (MWC215) which cannot synthesize NDH-2, the uncoupled NADH dehydrogenase. The data show a statistically significant decrease in the specific oxygen consumption rate for the strain lacking *ndh*. The inability to synthesize NDH-2 should force all of the electron flux from NADH through the coupled enzyme, NDH-1 (Fig. 1). Therefore, one might expect an increase in the bioenergetic efficiency, i.e., reduction in the O_2 consumption rate per gram of biomass, as a result of this mutation. This assumes, however, that (i) the wild-type strain uses NDH-2 to a significant extent in its respiration and (ii) the respiratory electron flux partitions through the two oxidases to the same extent in the two strains. If it is assumed that the wild-type strain is using primarily NDH-2 and the *bo*-type oxidase, then one would predict a large effect resulting from the *ndh* mutation due to the forced switch from NDH-2 to NDH-1 in strain MWC215. The specific oxygen consumption rate would be expected to drop to about 0.6 of the wild-type value. The observed effect is substantially smaller (0.9 of the wild-type value at high growth rates). The simplest interpretation is that in the wild-type strain, substantial electron flux is directed through NDH-1. This readily explains the relatively modest effect due to the elimination of NDH-2.

Comparison of strains utilizing the *bo*-type or *bd*-type oxidase. Strains GO103 and GO104 are isogenic, except that one can synthesize only the *bo*-type quinol oxidase (GO103) and the other contains only the *bd*-type quinol oxidase (GO104). The data in Fig. 3 show that at all growth rates, the specific oxygen consumption by the strain utilizing the *bo*-type oxidase (GO103) is less than that in the strain utilizing the *bd*-type oxidase (GO104). This is qualitatively compatible with the in vitro data (27) that show that the *bo*-type oxidase translocates more protons per electron than does the *bd*-type oxidase, i.e., it is more efficiently coupled. The fact that the growth properties of the strain lacking the *bd*-type oxidase are very similar to those of the wild-type suggests that most of the electron flux is through the *bo*-type oxidase in the wild-type strain. Note that this interpretation assumes that the partition of the electron flux through NDH-1 and NDH-2 is identical in the strains being compared. Quantitatively, if one assumes that the electron flux is equally divided between NDH-1 and NDH-2, switching from

the *bo*-type oxidase to the *bd*-type oxidase should result in an increase in the specific oxygen consumption rate by about 1.4-fold. The measured value (Fig. 4) varies from 1.45 to 1.13, depending on the growth rate. This may indicate an influence of the growth rate on the partitioning of the electron flux through the NADH dehydrogenases.

The comparison of strains MWC215 (*ndh*) and MWC217 (*ndh cyo*) also illustrates the effect due to elimination of the *bo*-type oxidase. The ratio of the specific oxygen consumption rates for strains MWC215 and MWC217 (Fig. 4) shows that the *cyo* mutation results in an increased respiration rate varying from 1.39- to 1.46-fold over the range of growth rates examined. The predicted effect is about a 1.3-fold increase if one assumes that the *bd*-type oxidase translocates $1H^+/e^-$, as measured by in vitro techniques (27), and about 1.9-fold if one assumes that the *bd*-type oxidase is uncoupled ($0H^+/e^-$). Hence, the experimental data are consistent with the conclusion that although the *bd*-type oxidase is less efficient than the *bo*-type oxidase, it is still coupled.

In summary, these experiments demonstrate effects on cell growth yield consistent with the expectations based on the in vitro proton translocation data (22, 27). The most clear-cut experiments are those with strains which are constrained to use only one NADH dehydrogenase and one terminal oxidase. The recent cloning of the *nuo* genes encoding the coupled NDH-1 enzyme (37) will facilitate the construction of the additional strains required for a more quantitative analysis.

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