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The Stationary-Phase-Exit Defect of *cydC* (*surB*) Mutants Is due to the Lack of a Functional Terminal Cytochrome Oxidase

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The *surB* gene was identified as a gene product required for *Escherichia coli* cells to exit stationary phase at 37°C under aerobic conditions. *surB* was shown to be the same as *cydC*, whose product is required for the proper assembly and activity of cytochrome *d* oxidase. Cytochrome *d* oxidase, encoded by the *cydAB* operon, is one of two alternate terminal cytochrome oxidases that function during aerobic electron transport in *E. coli*. Mutations inactivating the *cydAB* operon also cause a temperature-sensitive defect in exiting stationary phase, but the phenotype is not as severe as it is for *surB* mutants. In this study, we examined the phenotypes of *surB1* Δ*cydAB* double mutants and the ability of overexpression of cytochrome *o* oxidase to suppress the temperature-sensitive stationary-phase-exit defect of *surB1* and Δ*cydAB* mutants and analyzed spontaneous suppressors of *surB1*. Our results indicate that the severe temperature-sensitive defect in exiting stationary phase of *surB1* mutants is due both to the absence of terminal cytochrome oxidase activity and to the presence of a defective cytochrome *d* oxidase. Membrane vesicles prepared from wild-type, *surB1*, and Δ*cydAB* strains produced superoxide radicals at the same rate in vitro. Therefore, the aerobic growth defects of the *surB1* and Δ*cydAB* strains are not due to enhanced superoxide production resulting from the block in aerobic electron transport.

Aerobic electron transport in *Escherichia coli* functions with one of two alternate terminal oxidases, cytochrome *o* oxidase or cytochrome *d* oxidase (10). The two terminal oxidases have different biochemical properties and are expressed under different growth conditions. Cytochrome *o* oxidase, which is encoded by the *cyoABCDE* operon, predominates under highly aerobic conditions (23, 27, 31), while cytochrome *d* oxidase, which is encoded by the *cydAB* operon, predominates under microaerophilic conditions and is the predominant form of cytochrome oxidase present in stationary-phase cells (11, 27, 31). The ArcA-ArcB two-component regulatory system controls which terminal oxidase is present during aerobic growth by controlling expression of the *cyo* and *cyd* genes (21). Under low O_2 conditions, ArcA-ArcB activates transcription of *cydAB* and represses transcription of *cyo* and other genes involved in aerobic growth. ArcA and ArcB are not responding to O_2 concentration directly but instead may be directly or indirectly sensing the redox state of the electron transport chain (18, 21). Under anaerobic conditions, transcription of both *cydAB* and *cyoABCDE* is repressed by Fnr (6, 31).

Proper assembly and activity of cytochrome *d* oxidase requires the *cydD* and *cydC* gene products (12, 26). *cydD* and *cydC* mutants produce the apoproteins of the cytochrome *d* oxidase complex, but spectroscopic analysis indicates that the mutant complexes are missing one (*cydC*) (12) or all (*cydD*) (26) of the heme groups present in wild-type complexes. On the basis of the deduced amino acid sequence, *cydD* and *cydC* encode members of the ABC transporter family and have been proposed to export heme or some component required for assembly of heme into the cytochrome *d* oxidase complex (25).

The *cydC* gene was independently identified as *surB*, a gene product required for *E. coli* cells to exit stationary phase at 37°C under aerobic conditions (28). *cydAB* mutants also have a temperature-sensitive defect in exiting stationary phase, but the phenotype is not as severe as it is for *surB* mutants (28). This difference could indicate either that the *surB* gene product is required for the normal function of some other cellular component essential for exiting stationary phase or that the presence of a defective cytochrome *d* oxidase is more deleterious than its absence. To distinguish these possibilities, we examined the phenotypes of *surB* Δ*cydAB* double mutants and the ability of ectopic expression of cytochrome *o* oxidase to suppress the growth defects and analyzed spontaneous suppressors of *surB1*. Our results indicate that the severe stationary-phase-exit defect of *surB1* mutants is due to both the absence of a cytochrome terminal oxidase activity and the presence of a defective cytochrome *d* oxidase.

MATERIALS AND METHODS

Strains and plasmids used. The strains and plasmids used in this study are shown in Table 1. Transductions were done with bacteriophage P1*vir*. The presence of the *arcA1* or *arcB1* mutations in ZK126 was detected by sensitivity to toluidine blue (17).

Media. The minimal medium used was M63 medium (22) with 1 mM $MgSO_4$, 2 μg of thiamine per ml, and 10 mM KNO_3. The following carbon sources were added at the indicated concentrations: glucose (0.2%), glycerol (0.5%), and succinate (0.3%). When present, vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.) were added to a final concentration of 0.1%. Minimal medium plates contained 15 g of Difco BiTek agar per liter. The rich medium used was LB (22). LB plates contained 20 g of Difco BiTek agar per liter.

Determining EOP and colony size. Cultures were grown in tubes (18 by 150 mm) on a roller drum at 25°C in M63 glucose medium with 0.1% Casamino Acids. After 48 h, the stationary-phase cultures were diluted, aliquots were plated on either minimal glucose medium with Casamino Acids or LB medium, and the plates were incubated at 25 or 37°C. The plates incubated at 37°C were examined to determine the number of colonies and the colony diameter after 20 to 24 h, unless indicated otherwise. The plates incubated at 25°C were examined after 48 h, unless indicated otherwise. The efficiency of plating (EOP) at 37°C was the number of CFU per milliliter at 37°C divided by the number of CFU per milliliter at 25°C.

Preparation of membrane vesicles and measurement of NADH dehydrogenase activity and superoxide production in vitro. Cultures were grown at 30°C in M63 glucose medium with 0.1% Casamino Acids to stationary phase with extremely vigorous shaking. Oxygen saturation of the cultures was verified with a Clarke electrode (Rank Bros., Cambridge, United Kingdom). Some cells were serially diluted and plated aerobically and anaerobically to confirm the phenotype of the

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TABLE 1. *E. coli* K-12 strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
Strains		
ZK126	W3110 Δ*lacU169 tna-2*	R. Kolter (4)
ECL585	*arcA1 zjj*::Tn*10*	E. C. C. Lin (19)
ECL594	*arcB1 zgi*::Tn*10*	E. C. C. Lin (17)
ECL937	Δ(*cydAB′*)*455 zbg*::*kan* Φ(*cyo-lac*) *bla*$^+$	E. C. C. Lin (18, 24)
ECL946	Δ*cydAB*::*cam* Δ(*cyoABCDE*)*456*::*kan*	E. C. C. Lin (18, 24)
DS28	ZK126 *surB1*::miniTn*10kan*	28
DS126	ZK126 Δ(*cydAB′*)*455 zbg*::*kan*	ZK126 × P1(ECL937), (28)
DS127	ZK126 *surB1*::miniTn*10kan*	ZK126 × P1(DS28)
DS187	ZK126 Δ*cydAB*::*cam*	ZK126 × P1(ECL946)
DS188	ZK126 *surB1*::miniTn*10kan* Δ*cydAB*::*cam*	DS127 × P1(ECL946)
DS195	ZK126 *surB1*::miniTn*10kan arcA*$^+$ *zjj*::Tn*10*	DS127 × P1(ECL585)
DS196	ZK126 *surB1*::miniTn*10kan arcA1 zjj*::Tn*10*	DS127 × P1(ECL585)
DS197	ZK126 *surB1*::miniTn*10kan arcB*$^+$ *zgi*::Tn*10*	DS127 × P1(ECL594)
DS199	ZK126 *surB1*::miniTn*10kan arcB1 zgi*::Tn*10*	DS127 × P1(ECL594)
DS246	ZK126 *arcA*$^+$ *zjj*::Tn*10*	ZK126 × P1(ECL585)
DS247	ZK126 *arcA1 zjj*::Tn*10*	ZK126 × P1(ECL585)
DS248	ZK126 *arcB*$^+$ *zgi*::Tn*10*	ZK126 × P1(ECL594)
DS249	ZK126 *arcB1 zgi*::Tn*10*	ZK126 × P1(ECL594)
DS257	ZK126 Δ(*cydAB′*)*455 zbg*::*kan arcA*$^+$ *zjj*::Tn*10*	DS246 × P1(ECL937)
DS258	ZK126 Δ(*cydAB′*)*455 zbg*::*kan arcA1 zjj*::Tn*10*	DS247 × P1(ECL937)
DS259	ZK126 Δ(*cydAB′*)*455 zbg*::*kan arcB*$^+$ *zgi*::Tn*10*	DS248 × P1(ECL937)
DS260	ZK126 Δ(*cydAB′*)*455 zbg*::*kan arcB1 zgi*::Tn*10*	DS249 × P1(ECL937)
DS411	ZK126 pBR322	
DS412	ZK126 pRG110	
DS414	ZK126 Δ(*cydAB′*)*455 zbg*::*kan* pBR322	DS411 × P1(DS258)
DS416	ZK126 *surB1*::miniTn*10kan* pBR322	DS411 × P1(DS196)
DS418	ZK126 Δ(*cydAB′*)*455 zbg*::*kan* pRG110	DS412 × P1(DS258)
DS420	ZK126 *surB1*::miniTn*10kan* pRG110	DS412 × P1(DS196)
Plasmids		
pDT1-5	*sodA* in pHC79Ap^r Tc^r	D. Touati (30)
pRG110	*cyoABCDE* in pBR322 Ap^r	R. B. Gennis (1)

mutant cultures. For the experiment shown in Table 3, *surB1* cultures contained 0.2% revertant cells and Δ*cydAB*::*cam* cultures contained 3% revertant cells.

Inverted membrane vesicles were prepared and then assayed for NADH oxidase activity, NADH dehydrogenase II activity, and superoxide radical ($O_2^{-\cdot}$) production as described previously (15), except that the assays were done at 37°C. Briefly, NADH oxidation was monitored at 340 nm. NADH dehydrogenase II levels were determined by measuring NADH oxidation in the presence of 3.3 mM cyanide to block the cytochrome oxidases and 100 μM plumbagin, which directly reoxidizes the NADH dehydrogenase. To measure NADH oxidation by the electron transport chain, assays were done in the absence of cyanide and plumbagin. To assay superoxide radical production, membranes were diluted into 50 mM KPO_4 (pH 7.8) at 37°C in the presence of 30 μM ferricytochrome *c*. Parallel reactions contained either no CuZn superoxide dismutase (SOD) or 20 U of it. Reactions were initiated by the addition of 200 μM NADH. Superoxide was measured as the SOD-sensitive cytochrome *c* reduction by monitoring the A_{550}. The NADH, SOD, cytochrome *c*, and plumbagin used in the assays were from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS AND DISCUSSION

Δ*cydAB* is epistatic to *surB1*. *surB* and Δ*cydAB* mutants both have temperature-sensitive growth defects, but the phenotypes of *surB* mutants are more severe than those of Δ*cydAB* mutants (28). Both mutant strains have a temperature-sensitive defect in exiting stationary phase (Table 2A). Stationary-phase *surB1* cells have a greatly reduced efficiency of plating on minimal glucose medium at 37°C. In contrast, stationary-phase Δ*cydAB* cells have an efficiency of plating of 1 at 37°C but form much smaller colonies than the parental strain. The same temperature-sensitive defects in exiting stationary phase were seen if *surB1* or Δ*cydAB* cells entered stationary phase at either 25 or 37°C (data not shown). Prior to entering stationary phase, both mutants grew normally at 37°C on minimal glucose medium with Casamino Acids (reference 28 and data not shown). A second phenotype of both *surB1* and Δ*cydAB* mutants is that they are temperature sensitive for growth on LB medium in both exponential phase and stationary phase (reference 32 and data not shown). Again, the phenotype of *surB1* mutant cells was more severe than that of Δ*cydAB* mutant cells (Table 2A). Exponential-phase Δ*cydAB* cells formed small colonies on LB medium at 37°C, while exponential-phase *surB1* cells formed pinpoint colonies that were not visible for 36 to 48 h. These remained as pinpoint colonies even after several days of incubation at 37°C.

We constructed a *surB1* Δ*cydAB* double mutant and found that its phenotypes were nearly indistinguishable from those of the Δ*cydAB* mutant (Table 2A). These results show that the defect in *cydAB* is epistatic to the defect in *surB1*. Thus, loss of cytochrome *d* oxidase activity is sufficient to explain the stationary-phase-exit defect of *surB1* cells. The more severe growth defect of *surB1* mutants indicates that the presence of the defective cytochrome *d* oxidase is more deleterious than its absence.

Increased expression of cytochrome *o* oxidase suppressed the growth defects of the *surB1* mutant. Two of four spontaneous suppressors of *surB1* were mapped by P1 transduction to the region of the chromosome that includes *arcA* (data not shown). To test whether known mutations in either *arcA* or *arcB* suppressed the stationary-phase-exit defect of *surB1*, we introduced known *arcA* or *arcB* alleles into the *surB1* strain. Double mutants containing *surB1* and either *arcA1* or *arcB1* were able to resume growth at 37°C under aerobic conditions on minimal glucose medium (Table 2B). *arcA1* or *arcB1* also suppressed the LB sensitivity of *surB1* cells at 37°C (Table 2B). On LB medium at 37°C, *surB1 arcA1* and *surB1 arcB1* cells

TABLE 2. Effects of Δ*cydAB* and *arc* mutations and multicopy *cyoABCDE* on plating of *surB1* strains[a]

Strain	Relevant genotype	M63 glucose medium + Casamino Acids		LB medium	
		EOP[d]	Colony diam (mm)	EOP	Colony diam (mm)
A. Epistatic interactions					
ZK126	Wild type	1.2	1.2	1.5	2.5
DS127	*surB1*	$<2.0 \times 10^{-5}$	—[b]	1.0	Pinpoint[c]
DS187	Δ*cydAB*::*cam*	1.0	0.1	0.8	0.1
DS188	*surB1* Δ*cydAB*::*cam*	0.9	0.1	0.6	<0.1
B. Introduction of *arc* mutations					
ZK126	Wild type	0.9	1	1.0	2–2.5
DS195	*surB1 arcA*$^+$ *zjj*::Tn*10*	1.2×10^{-3}	1	0.8	Pinpoint[c]
DS196	*surB1 arcA1 zjj*::Tn*10*	0.8	1	1.0	2
DS197	*surB1 arcB*$^+$ *zgi*::Tn*10*	1.1×10^{-3}	1	1.6	Pinpoint[c]
DS199	*surB1 arcB1 zgi*::Tn*10*	1.0	1	0.8	1.5
DS257	Δ(*cydAB′*)*455 arcA*$^+$ *zjj*::Tn*10*	1.3	0.2	1.4	0.2
DS258	Δ(*cydAB′*)*455 arcA1 zjj*::Tn*10*	0.7	1	1.3	2
DS259	Δ(*cydAB′*)*455 arcB*$^+$ *zgi*::Tn*10*	1.0	0.2	0.9	0.2
DS260	Δ(*cydAB′*)*455 arcB1 zgi*::Tn*10*	0.7	1	0.7	2
C. Multicopy *cyo* operon					
DS411	Wild type/pBR322	0.7	1.5	1.4	3
DS412	Wild type/pRG110	0.8	1.5	1.1	2
DS416	*surB1*/pBR322	1.6×10^{-3}	1	0.9	Pinpoint[c]
DS420	*surB1*/pRG110	0.6	1.5	0.6	0.5–1[e]
DS414	Δ(*cydAB′*)*455*/pBR322	0.7	0.1	1.1	0.2
DS418	Δ(*cydAB′*)*455*/pRG110	1.9	1.5	0.8	1.8

[a] Cultures were grown and EOP and colony diameter at 37°C were determined as described in Materials and Methods. For each section, the data shown are from a representative experiment. Each strain was independently assayed at least twice.
[b] In this experiment, no *surB1* colonies grew at 37°C in the least-diluted sample plated. In other experiments, the *surB1* colonies that grew at 37°C had the same diameter as that of wild-type colonies and were presumably revertants.
[c] Pinpoint colonies formed by *surB1* cells on LB medium were not visible until 36 to 48 h of incubation at 37°C.
[d] The *surB1* colonies that grew at 37°C were presumably revertants.
[e] Approximately 50% of the colonies were 0.5 mm in diameter and 50% were 1 mm in diameter.

formed colonies that were only slightly smaller than those formed by wild-type cells.

Introducing *arcA1* or *arcB1* mutations into the Δ*cydAB* strain also suppressed the stationary-phase-exit defect of this strain (Table 2B). As reported previously (32), we also found that introducing *arcA1* or *arcB1* alleles into the Δ*cydAB* strain suppressed the temperature-sensitive growth defect on LB medium at 37°C (Table 2B).

arcA and *arcB* mutations should derepress expression of numerous gene products active during aerobic growth, including cytochrome *o* oxidase (20). To determine whether increased expression of cytochrome *o* oxidase was sufficient to suppress *surB1*, we introduced pRG110 into the *surB1* mutant strain. Cells containing plasmid pRG110, which carries the *cyoABCDE* operon, have almost fivefold-higher levels of cytochrome *o* oxidase than cells without the plasmid (1). The presence of pRG110 allowed *surB1* cells to resume growth at 37°C under aerobic conditions on minimal glucose medium (Table 2C). As a control, the vector plasmid pBR322 did not suppress the stationary-phase-exit defect. Introduction of pRG110 also partially suppressed the temperature-sensitive growth defect on LB at 37°C (Table 2C). *surB1* cells containing pBR322 formed pinpoint colonies after 48 h of growth on LB medium at 37°C, while *surB1* cells containing pRG110 formed significantly larger colonies. However, these colonies were still smaller than those formed by the wild-type strain. Introduction of plasmid pRG110 into the Δ*cydAB* strain suppressed the stationary-phase-exit defect of this mutant on minimal glucose medium (Table 2C). As reported previously (32), introducing pRG110 also suppressed the temperature-sensitive growth defect on LB medium at 37°C (Table 2C).

These results indicate that *E. coli* requires a terminal oxidase to exit from stationary phase under aerobic conditions at 37°C. In fact, we believe this reflects the general requirement for respiration for *E. coli* to grow rapidly in the presence of oxygen (33). A variety of mutants in which aerobic electron transport is blocked grow very poorly in the presence of oxygen (1, 3, 18, 33, 34). The defect in *surB1* and Δ*cydAB* strains is specific for exiting from stationary phase, because these are conditions where the mutant cells are deficient in cytochrome oxidase activity despite the presence of intact *cyo* genes. Transcription of *cyoABCDE* is repressed and transcription of *cydAB* is activated as batch cultures approach stationary phase (11, 23). Therefore, when cells resume growth, the major terminal oxidase present is cytochrome *d* oxidase. Once exponential growth has started, *surB1* and *cydAB* strains grow normally at high temperature because *cyoABCDE* is expressed and active cytochrome *o* oxidase is present.

Stationary-phase membranes from *surB1*, Δ*cydAB*, and wild-type cells produced the same amounts of superoxide in vitro. The ability of cytochrome *o* oxidase to suppress the *surB1* defect shows that it is the loss of terminal electron acceptor activity that accounts for the stationary-phase-exit defect rather than some other specialized function of cytochrome *d* oxidase. This raised the possibility that the stationary-phase-exit defect is due to oxidative stress caused by increased production of reactive oxygen species when electron transport is blocked by the absence of a functional terminal oxidase. En-

TABLE 3. Superoxide production and NADH dehydrogenase activity in inverted membrane vesicles[a]

Strain	Relevant genotype	NADH oxidase[b]	NdH II activity[c]	$O_2^{-\cdot}$ production[d]
ZK126	Wild type	10.3	17.6	7.2
DS127	*surB1*::miniTn*10kan*	4.0	23.8	6.6
DS186	Δ*cydAB*::*cam*	2.6	26.9	7.9

[a] Membrane vesicles were prepared and superoxide production and NADH dehydrogenase activity were assayed as described in Materials and Methods. Each assay was repeated at least twice. The data shown are representative of the results obtained with several independent preparations of membrane vesicles.
[b] Micromoles of NADH oxidized per minute per membranes of 10^{12} cells.
[c] Micromoles of NADH oxidized by the NADH:plumbagin oxidoreductase activity of NADH dehydrogenase II (NdH II) per minute per membranes of 10^{12} cells.
[d] Nanomoles of superoxide radical ($O_2^{-\cdot}$) formed per minute per membranes of 10^{12} cells in the presence of NADH.

hanced production of superoxide radicals and hydrogen peroxide by membrane vesicles has been observed in vitro when the aerobic electron transport chain is blocked by inhibitors (13, 16). Because the primary effect of blocking electron transport is enhanced production of superoxide radicals, which subsequently dismutate to form H_2O_2 (8, 13), we focused on superoxide production as a possible cause of the stationary-phase-exit defect in *surB1* and Δ*cydAB* cells.

If superoxide production is responsible for the stationary-phase-exit defect, it might be possible to suppress the defect by overproducing SOD. The presence of the *sodA* gene on a multicopy plasmid leads to four- to fivefold overexpression of the manganese SOD (30). Introduction of pDT1-5 into *surB1* or Δ*cydAB* mutants did not suppress either the stationary-phase-exit defect or the LB medium sensitivity at 37°C (data not shown). These experiments were done in Mn-supplemented medium (100 μM $MnCl_2$) to satisfy the metal cofactor requirement of the enzyme.

This result indicated that either superoxide production was not responsible for the stationary-phase-exit defect or that the amount of superoxide produced was high enough to overcome the ability of the increased SOD levels to detoxify it. To examine the second possibility, we measured superoxide production in vitro by inverted membrane vesicles prepared from *surB1*, Δ*cydAB*, and wild-type cells. Membrane vesicles prepared from *surB1* and Δ*cydAB* cells had lower NADH oxidase activity than membrane vesicles from the wild-type strain, presumably because of the lack of cytochrome *d* oxidase in the mutant strains (Table 3). Mutant and wild-type strains contained comparable levels of NADH dehydrogenase II (Table 3), showing that the observed differences in NADH oxidase activity were not due to differences in abundance of this enzyme. Total superoxide production levels by the three strains were indistinguishable (Table 3). However, the two mutant strains produced two to four times as much superoxide radicals per micromole of NADH oxidized as the wild-type strain.

The stationary-phase-exit defect of *surB1* cells at 37°C is suppressed by growth on glycerol or succinate. Why is the stationary-phase-exit defect of *surB* and Δ*cydAB* mutants seen only at high temperature? One possible explanation is that another terminal cytochrome oxidase, such as that encoded by the *cyxAB* genes (7, 29), is active at 25°C but not at 37°C. This seems unlikely because mutants lacking both cytochrome *o* and cytochrome *d* oxidase (Δ*cydAB* Δ*cyoABCDE*) did not grow on the nonfermentable carbon sources glycerol and succinate at 25 or 37°C (data not shown).

Another possibility is that at the low growth rate at 25°C, cells are able to cope with the lack of cytochrome *d* oxidase. This model predicts that the 37°C stationary-phase-exit defect should be alleviated by growth on media that support a low growth rate. To test this hypothesis, we measured the plating efficiency of stationary-phase cells of wild-type and *surB1* cultures on minimal medium with glycerol and succinate as carbon sources (Table 4). When 0.1% Casamino Acids was present in the medium, growth on glycerol only partially suppressed the stationary-phase-exit defect, but growth was completely normal on minimal glycerol medium lacking Casamino Acids. Stationary-phase *surB1* cells grew normally at high temperature on minimal succinate medium both with and without 0.1% Casamino Acids.

Unlike the results of Wall et al. (32), our results showed that the presence of 0.4% glucose did not suppress either the temperature-sensitive stationary-phase-exit defect on minimal glucose medium or the LB medium sensitivity of *surB1* or Δ*cydAB* strains (data not shown). This difference may be due to differences in the strain backgrounds used.

The reason *E. coli* cells need cytochrome oxidase to grow aerobically is not clear. One possible explanation for the aerobic growth defects of *surB1* and Δ*cydAB* strains is that growth is being inhibited by the production of reactive oxygen species. We show here that superoxide production is not increased in either *surB1* or Δ*cydAB* cells. However, the growth defects observed could still be due to higher production of another toxic oxygen species, such as hydrogen peroxide. In addition, the mutant strains may be less able to cope with levels of

TABLE 4. Suppression of the stationary-phase-exit defect of *surB1* and Δ*cydAB* strains by growth on glycerol or succinate[a]

Plating medium	WT (ZK126)		*surB1* (DS127)		Δ(*cydAB′*)*455* (DS126)	
	EOP	Colony diam (mm)	EOP[c]	Colony diam (mm)	EOP	Colony diam (mm)
M63 glucose + CAA[b]	0.7	1.2	1.0×10^{-4}	0.2	1.5	0.2
M63 glycerol + CAA	0.8	0.75	0.04	0.5	2.1	0.5
M63 succinate + CAA	1.0	0.5	2.1	0.5	1.0	0.5
M63 glucose	1.3	2.0[d]	1.3×10^{-4}	2.0	0.5	1.0
M63 glycerol	0.8	1.2[d]	1.0	0.75	0.3	0.75
M63 succinate	1.0	0.2[d]	0.8	0.2	0.7	0.2

[a] Cultures were grown and EOPs and colony diameters at 37°C were determined as described in Materials and Methods. The data shown are from a representative experiment. Each strain was independently assayed at least twice.
[b] CAA, 0.1% Casamino Acids.
[c] The *surB1* colonies that grew at 37°C were presumably revertants.
[d] Colony diameters were measured after 48 h of incubation at 37°C. For comparison, colony diameters after 24 h at 37°C on M63 glucose medium were 1 mm for ZK126, 0.5 mm for DS127, and 0.2 mm for DS126.

whatever reactive oxygen species are being produced, because full expression of catalase and MnSOD appear to be dependent on a functional electron transport chain (14, 18, 21).

A second possible explanation for the requirement for cytochrome oxidase activity during aerobic growth is that cells need a terminal oxidant to ensure that NADH is reoxidized. The pathways that reoxidize NADH during anaerobic fermentation of glucose are not present in aerobically grown cells (2). Accumulation of NADH could inhibit central metabolism by inhibiting the activity of enzymes such as glyceraldehyde-3-phosphate dehydrogenase and pyruvate dehydrogenase, which require NAD^+ (9). This model could account for both the temperature sensitivity and the carbon source dependence of the growth defects. NADH production during growth on minimal glucose or LB medium will be more rapid at 37°C than at 25°C. Similarly, NADH production will be slower during growth on glycerol or succinate at 37°C than during growth on glucose or LB medium at the same temperature.

The aerobic growth defects are more severe in the *surB* mutant than in an isogenic *surB* Δ*cydAB* mutant, indicating that the defective cytochrome *d* oxidase produced in the *surB* mutant is deleterious to *E. coli*. The molecular basis for this toxicity is not clear. Some conditions that suppress the *surB* phenotypes, such as mutations in *arcA* or *arcB* or introduction of multicopy plasmids carrying the *cyo* operon, should increase the level of cytochrome *o* oxidase. Plating on minimal glycerol or succinate medium could also lead to higher levels of cytochrome *o* oxidase, since expression of the *cyoABCDE* operon is two- to threefold higher during growth on glycerol or succinate than during growth on glucose (5, 23). In addition to satisfying the requirement for an intact aerobic electron transport chain as discussed above, increased production of cytochrome *o* oxidase might also alleviate the toxic effects of the defective cytochrome *d* oxidase. For example, by competing for reduced quinones, cytochrome *o* oxidase might minimize electron flux to cytochrome *d* oxidase.

Alternatively, reduction of *cydAB* expression may be sufficient to ameliorate the toxicity of the defective cytochrome *d* oxidase. Mutations in *arcA* or *arcB* should decrease the level of the defective cytochrome *d* oxidase, since ArcA is an activator of *cydAB* transcription in addition to being a repressor of the *cyo* operon. While we have not measured *cydAB* expression or accumulation of the defective cytochrome *d* oxidase in strains where the *cyo* operon is carried on a multicopy plasmid, it is possible that titration of ArcA by the plasmid could decrease the amount of activator available to act at the *cydAB* promoter.

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