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Mutations in *Escherichia coli* That Effect Sensitivity to Oxygen

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Fifteen oxygen-sensitive (Oxy^s) mutants of *Escherichia coli* were isolated after exposure to UV light. The mutants did not form macroscopic colonies when plated aerobically. They did form macroscopic colonies anaerobically. Oxygen, introduced during log phase, inhibited the growth of liquid cultures. The degree of inhibition was used to separate the mutants into three classes. Class I mutants did not grow after exposure to oxygen. Class II mutants were able to grow, but at a reduced rate and to a reduced final titer, when compared with the wild-type parent. Class III mutants formed filaments in response to oxygen. Genetic experiments indicated that the mutations map to six different chromosomal regions. The results of enzymatic assays indicated that 7 of the 10 class I mutants have low levels of catalase, peroxidase, superoxide dismutase, and respiratory enzymes when compared with the wild-type parent. Mutations in five of the seven class I mutants which have the low enzyme activities mapped within the region 8 to 13.5 min. P1 transduction data indicated that mutations in three of these five mutants, Oxy^s-6, Oxy^s-14, and Oxy^s-17, mapped to 8.4 min. The correlation of low enzyme levels and mapping data suggests that a single gene may regulate several enzymes in response to oxygen. The remaining three class I mutants had wild-type levels of catalase, peroxidase, and superoxide dismutase, but decreased respiratory activity. The class II and III mutants had enzyme activities similar to those of the wild-type parent. Our results demonstrate that mutations in at least six genes can be expressed as oxygen sensitivity. Some of these genes may be involved in respiration or cell division or may regulate the expression of several enzymes.

Oxygen and hyperbaric oxygen have been shown to cause mutations (7, 15, 19, 45). Cells metabolizing in the presence of oxygen can produce various oxygen radicals. These reactive oxygen species can cause mutations (14, 25, 31), initiate lipid peroxidation (30), oxidize proteins (6), and induce *Escherichia coli* DNA repair pathways (11, 14). Various models have been proposed to account for oxygen toxicity in procaryotes (32). By-products of aerobic metabolism (i.e., superoxide radical, hydrogen peroxide) and the action of oxygen on various cellular components and on the surrounding medium have been proposed as mechanisms by which oxygen may be toxic to cells (32).

Recently, superoxide dismutase (SOD) has been proposed as a major defense against the toxic effects of oxygen (16). A double mutant of *E. coli* lacking both the iron-containing (FeSOD) and manganese-containing (MnSOD) SODs is able to grow aerobically, but at a reduced rate, in complex liquid medium (8). The double mutant is extremely sensitive to the superoxide radical-generating compound paraquat (8) and shows an increase in mutation rate when grown aerobically (13). The results suggest that SOD is required for maximal growth aerobically and for protection from the mutagenic effects of oxygen, but that additional defenses from oxygen toxicity must be present to account for the aerobic growth of the double mutant.

Resistance to H_2O_2 has been shown to be induced in *E. coli* (11), and the resistance involves a DNA repair pathway. An H_2O_2-resistant *Salmonella typhimurium* mutant constitutively overexpresses nine proteins. These proteins are positively regulated by the *oxyR* gene (9). In sum, these studies indicate that enzymes in addition to catalase and SOD can be induced to protect cells against oxidative damaging agents.

Previous studies have reported mutations in *E. coli* (20) and *S. typhimurium* (44) that resulted in oxygen intolerance. In *E. coli*, these mutations involved the loss of either of two groups of enzymes: catalase and peroxidase, or catalase, peroxidase, and MnSOD (20). In *S. typhimurium*, loss of topoisomerase I activity resulted in mutants that would only grow anaerobically (44). Yamamoto and Droffner suggested that the supercoiled state of the genome regulated the expression of several enzymes required for aerobic growth (44). Each of these studies indicate that several enzymes are probably required for maximum oxygen tolerance.

To further investigate the mechanisms of oxygen toxicity, 15 oxygen-sensitive (Oxy^s) mutants of *E. coli* K-12 strain AB1157 have been isolated. These mutants do not form colonies when plated aerobically, but do form colonies anaerobically. In this contribution we present and interpret genetic and biochemical data on this group of mutants.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The oxygen-tolerant *E. coli* K-12 Hfr, Tn*10* insertion, and F-prime strains listed in Table 1 were obtained from Barbara Bachmann of the *E. coli* Genetic Stock Center at Yale University. The oxygen-tolerant *E. coli* K-12 AB1157 parent strain is stocked in this laboratory. Each of the Oxy^s mutants isolated retained the following genetic characteristics of the parent strain (AB1157): *thr*, *leu*, *pro*, *his*, *arg*, *thi*, and Str^r. P1 *vir* was obtained from the laboratory of Hosni Hassan at North Carolina State University.

Media and buffers. The liquid complex medium, NYG, contained (per liter) 8 g of nutrient broth (Difco Laboratories), 5 g of yeast extract (Difco), 4 g of D-glucose, and 6 g of

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TABLE 1. Bacterial strains

Strain	Sex	Genetic characteristics	Origin of transfer (min)	Direction of transfer[a]
H	Hfr	λ⁻ *relA1 spoT1 thi-1* PO1 of HfrHayes	97	Cl
BW113	Hfr	*metB1 relA1* λ⁻ *spoT1* PO3 of HfrP4X	7	CC
KL226	Hfr	*relA1 tonA22* T2ʳ λ⁻*pit-10 spoT1* PO2A of HfrCavalli	13	Cl
KL14	Hfr	*thi-1 relA1* λ⁻ *spoT1* PO68	67	Cl
KL228	Hfr	*thi-1 leuB6 gal-6 lacY1* or *lacZ4* λ⁻ *supE44* PO13 of AB313	84.5	CC
Broda 8	Hfr	*metB1 relA1* λʳ λ⁻ *spoT1* PO118	8	Cl
KL209	Hfr	*thi-1 malB6* λʳ λ⁻ *supE44* PO18 of HfrJ4(P10)	91.5	CC
KL208	Hfr	*relA1*? λ⁻ PO43 of Broda 7	34.5	CC
KL983	Hfr	*xyl-7 lacY1 mglP1* λ⁻ PO53 of KL98	51.5	CC
KL99	Hfr	*thi-1 relA1 lac-42* λ⁻ *spoT1* PO42	22	Cl
KL96	Hfr	*thi-1 relA1* λ⁻ *spoT1* PO44	47	CC
KL25	Hfr	*supE42* λ⁻ PO46	82.5	Cl
KL16	Hfr	*thi-1 relA1* λ⁻ *spoT1* PO45	62	CC
PK191	Hfr	Δ(*gpt-lac*)*5 supE44 relA1*? *thi-1* λ⁻ PO66 of HfrPK19	42	Cl
Ra-2	Hfr	*mal-28 sfa-4* λʳ λ⁻ *supE42* PO48	88	Cl
P801	Hfr	*lacY1* or *lacY40 xyl-7 mtl-2* λ^ind⁻ *ara-41* PO120	3	CC
PK3	Hfr	*thr-1 leuB6 thi-1 lacY1 azi-15 tonA21* λ⁻ *supE44* PO131	77	Cl
AB1157	F⁻	*thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1 tsx-33*(T6ʳ) *tonˢ* λʳ *strʳ*		
JP3123	F⁻	*lacY1 aroL478*::Tn*10 tsx-67? purE42* λ⁻ *tyrR366 rpsL109 xyl-5 mtl-1 thi-1?*		
χ2844	F⁻	*tsx-462*::Tn*10* λ⁻		
W3747	F′	F13(*argF*⁺ *lac*⁺ *tsx-69 purE*⁺) *relA1 spoT1 metB1*, and deletion corresponding to F13		

[a] Cl, Clockwise; CC, counterclockwise, on the *E. coli* genetic map (4).

NaCl. For plates, 23 g of nutrient agar (Difco) per liter was substituted for nutrient broth. The liquid complex medium, LBG, contained (per liter) 10 g of tryptone (Difco), 5 g of yeast extract (Difco), 4 g of D-glucose, and 6 g of NaCl. The synthetic medium was a modified M9 medium (28), pH 7.4, containing the following (per liter): 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 2 g of NH_4Cl, 2 ml of 1 M $MgSO_4$, 0.4 g of D-glucose, amino acids (final concentrations) L-Thr (100 μg/ml), L-Leu (100 μg/ml), L-Pro (400 μg/ml), L-His (20 μg/ml), L-Arg (40 μg/ml), and thiamine (17 μg/ml), and 1 ml of trace elements solution (20.1 g of EDTA, 0.5 g of $CaCl_2$, 16.7 g of $FeCl_3 \cdot 6H_2O$, 0.18 g of $ZnSO_4 \cdot 7H_2O$, 0.1 g of $CuSO_4$, 0.18 g of $CoCl_2 \cdot 6H_2O$ per liter). For plates, Difco agar was added to 1.5%. When antibiotics were required, streptomycin sulfate was added to a final concentration of 200 μg/ml and tetracycline was added to a final concentration of 10 μg/ml. When needed, menaquinone, ubiquinone, and *p*-hydroxybenzoate were added to NYG medium at final concentrations of 1, 10, and 10 μg/ml, respectively. Phosphate buffer (0.067 M) contained 4.5 g of KH_2PO_4 and 5.8 g of K_2HPO_4 (anhydrous) per liter. SOD assay buffer was 50 mM potassium phosphate plus 0.1 mM EDTA, pH 7.8.

Culture conditions and anaerobic methods. All cultures were grown at 37°C. For isolation of mutants, uninoculated plates were incubated overnight in a vacuum to reduce their oxygen content. In all experiments, inoculated plates were incubated anaerobically in a Torbal anaerobic jar. Each jar was sparged at least three times with high-purity N_2 containing 5% CO_2. Liquid cultures were incubated anaerobically in medium containing an oxygen-consuming membrane fraction (P2) of *E. coli* (1, 2). For some experiments, liquid cultures in NYG or LBG were bubbled with high-purity N_2 to achieve anaerobiosis. Mutants were stocked in Bellco tubes containing NYG and P2. The medium was sparged three times with N_2 to lower the oxygen tension and sterilized, and, to achieve complete anaerobiosis, P2 was added 5 min before inoculation.

Method of mutagenesis and verification of genetic markers. All mutants were isolated after exposure to UV (254 nm) light. An overnight culture of *E. coli* AB1157 was diluted 1/1,000 into 0.067 M phosphate buffer and exposed to 44, 48, or 52 J of UV light per m^2 from two 15-W germicidal lamps. The irradiated suspension was plated onto NYG and anaerobically incubated overnight at 37°C in a Torbal anaerobic jar. The plates were then replica plated and returned to the anaerobic jar. The replicas were incubated aerobically at 37°C overnight. Pairs of plates were compared, and colonies that did not grow aerobically were restreaked. The isolates were temporarily stocked as stabs in NYG agar containing the P2 membrane fraction. Isolates were then checked for retention of genetic markers on M9 medium containing appropriate supplements and stocked in liquid NYG in Bellco tubes.

Microcolony formation. Cells were plated onto a thin layer of agar on microscope slides. The slides were incubated in air, 100% O_2, or 95% N_2–5% CO_2 for 6 h. Microcolonies were observed with a phase-contrast Zeiss photomicroscope.

Genetic mapping. All of the mutations were initially mapped by using Hfr strains and the rapid-mapping technique of Low (26) except that the print matings were incubated anaerobically for 30 min at 37°C. For transductional mapping, liquid cultures of JP3123 (*aroL478*::Tn*10*) and χ2844 (*tsx-462*::Tn*10*) (Table 1) were lysed with P1 *vir* as described by Silhavy et al. (34). With these lysates, transductions were performed essentially by the method of

Silhavy et al. (34) with cultures grown anaerobically in LBG supplemented with 5 mM $CaCl_2$ to late log phase (approximate optical density at 540 nm [OD_{540}] of 0.7). Phage were adsorbed to these cells at a multiplicity of infection of 1:1 during a 20-min incubation at 37°C. Sodium citrate was added (final concentration, 0.5 M) to prevent phage readsorption. LBG and P2 were added and the mixture was incubated for 75 min at 37°C to allow expression of tetracycline resistance (Tet^r). For F-prime complementation experiments, strain W3747 (Table 1) carrying a primary F-prime that contained approximately the 6- to 12.5-min region of the *E. coli* chromosome was used. Loopfuls of overnight cultures of Oxy^s-2, Oxy^s-3, Oxy^s-6, Oxy^s-14, and Oxy^s-17 were streaked directly onto M9 medium lacking methionine, but containing all of the supplements required for AB1157, a limiting amount of Difco yeast extract (0.01%), and 0.2% lactose as the sole fermentable carbon source. W3747 was streaked directly onto each of the individual streaks of the Oxy^s mutants to allow mating to occur. This procedure selects for Lac^+ and oxygen tolerance (Oxy^r) and selects against the Met^- F-prime donor. The plates were incubated aerobically for 2 days to select against the growth of the Oxy^s mutants and scored for complementation of the Lac^- and Oxy^s phenotypes.

Growth curves. Growth of liquid cultures was followed by measuring OD_{540}, using a Bausch & Lomb Spectronic 20 spectrophotometer. Anaerobic cultures were grown by bubbling 100% N_2 through the medium continuously. When the OD_{540} reached approximately 0.2, air or 100% O_2 was substituted for the N_2 for approximately 4 h. Mutant Oxy^s-2 does not grow as well anaerobically as the parent strain, so the study of the inhibitory effects was initiated at an OD_{540} of 0.1.

Growth of cells for enzyme assays and pyridine hemochrome determinations. Cells were grown anaerobically at 37°C in 4 liters of complex medium containing P2. The headspace was sparged with 100% N_2 and the containers were tightly capped. Oxygen-exposed cells were initially grown anaerobically in 400-ml culture volumes to late log phase and then exposed to 100% O_2 for 3 h. Cells were collected by centrifugation, washed once with SOD assay buffer, and frozen at −80°C until used.

Preparation of cell extracts and enzyme assays. Frozen cell pellets were thawed and suspended in SOD assay buffer. The cell suspensions were subjected to sonication at 4°C for 10 min in 30-s pulses, using a Branson Sonifier. The cell debris was removed by centrifugation, and the supernatant was dialyzed overnight against 50 mM potassium phosphate–0.1 mM EDTA, pH 7.8. The dialyzed extract was then cleared by centrifugation, and the supernatant was stored on ice until assayed. Catalase activity was measured by the method of Beers and Sizer (5). Peroxidase was measured by using the *o*-dianisidine procedure described in the *Worthington Enzyme Manual* (42). SOD was measured by the method of McCord and Fridovich (29). A unit of enzyme activity results in a 50% inhibition in the rate of reduction of cytochrome *c* by xanthine/xanthine oxidase. Protein was measured by the method of Lowry et al. (27). Oxygen uptake by whole cells was measured with a Gilson Oxygraph 5/6 equipped with a Clark-style oxygen electrode. For these measurements, cells were grown in flasks bubbled with 100% N_2, harvested by centrifugation and suspended in 0.067 M phosphate buffer. Oxygen uptake in 0.067 M phosphate buffer supplemented with 0.4% D-glucose is reported as percentage of the total oxygen removed per minute per milligram (dry weight) of cells. Cell samples (50 μl) were dried over phosphoric anhydride (P_2O_5) for at least 3 days for dry-weight determination.

Pyridine hemochrome and porphyrin determination. Membrane fractions were prepared from anaerobically grown cells. Frozen cell pellets were suspended in a ratio of 1 g (wet weight) of cells/2 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid), pH 7.5. The cell suspensions were subjected to sonication for at least 3 min in 30-s pulses, using an MSE sonicator. The extract was cleared by centrifugation (36,600 × *g*) for 30 min. The supernatant was then subjected to centrifugation at 60,000 × *g* overnight. The pellet, containing the membrane fraction, was frozen at −80°C until used. Extraction of pyridine hemochromes was performed by the method of Fuhrhop and Smith (18). Pyridine hemochrome dithionite reduced minus oxidized spectra were determined with a modified Cary-14 spectrophotometer (OLIS Instrument Co., Jefferson, Ga.). The concentrations of type *b* cytochromes in cytoplasmic membrane preparations from AB1157, Oxy^s-9, Oxy^s-10, and Oxy^s-12 were calculated from the pyridine hemochrome spectra, using the molar extinction coefficient for cytochrome b_1 of Jones and Redfearn (22). Total protein was determined by the biuret method (24).

For extraction and quantitation of accumulated porphyrins, AB1157 and Oxy^s-16 were cultured in NYG in flasks bubbled with N_2. Cells were harvested by centrifugation and suspended in 0.067 M phosphate buffer. Samples were dried over P_2O_5 for dry-weight determination. The remaining sample was subjected to extraction, using the ether-cyclohexanone method detailed by Sasarman et al. (33). The Soret peak was identified with a Hitachi model 110 spectrophotometer, and the quantity of porphyrins extracted per gram (dry weight) of cells was determined by using the correction factor for uroporphyrin given by Fuhrhop and Smith (17).

RESULTS

General characteristics of the Oxy^s mutants. The 15 Oxy^s mutants were obtained from a group of 142,954 colonies examined for ability to grow both aerobically and anaerobically. The mutation frequency for Oxy^s mutants was calculated to be approximately 10^{-4}.

All of the mutants retained the amino acid requirements of the parental strain AB1157. The mutants formed macroscopic colonies when plated anaerobically. They did not form macroscopic colonies when plated and incubated aerobically. However, if plates that had been incubated aerobically for as long as 24 h were subsequently incubated anaerobically, macroscopic colonies were produced. This indicated that oxygen was bacteriostatic rather than bacteriocidal for each of these mutants.

Although all mutants failed to form macroscopic colonies when incubated aerobically, they differed in ability to undergo limited growth and division under aerobic conditions. To study microcolony formation, cells were incubated aerobically on complex medium and observed by phase-contrast microscopy for a 6-h period. Oxy^s-3, Oxy^s-4, and Oxy^s-11 did not divide at all, Oxy^s-13 formed long nonseptate filaments, and the remaining mutants were capable of one to four divisions.

The mutants were assigned to phenotypic classes based on their growth behavior in liquid cultures. All of the mutants, except Oxy^s-2 and Oxy^s-9, grew as rapidly and to the same final OD_{540} as AB1157 under anaerobic conditions. Oxy^s-2 and Oxy^s-9 did not achieve the same final titer as the wild-

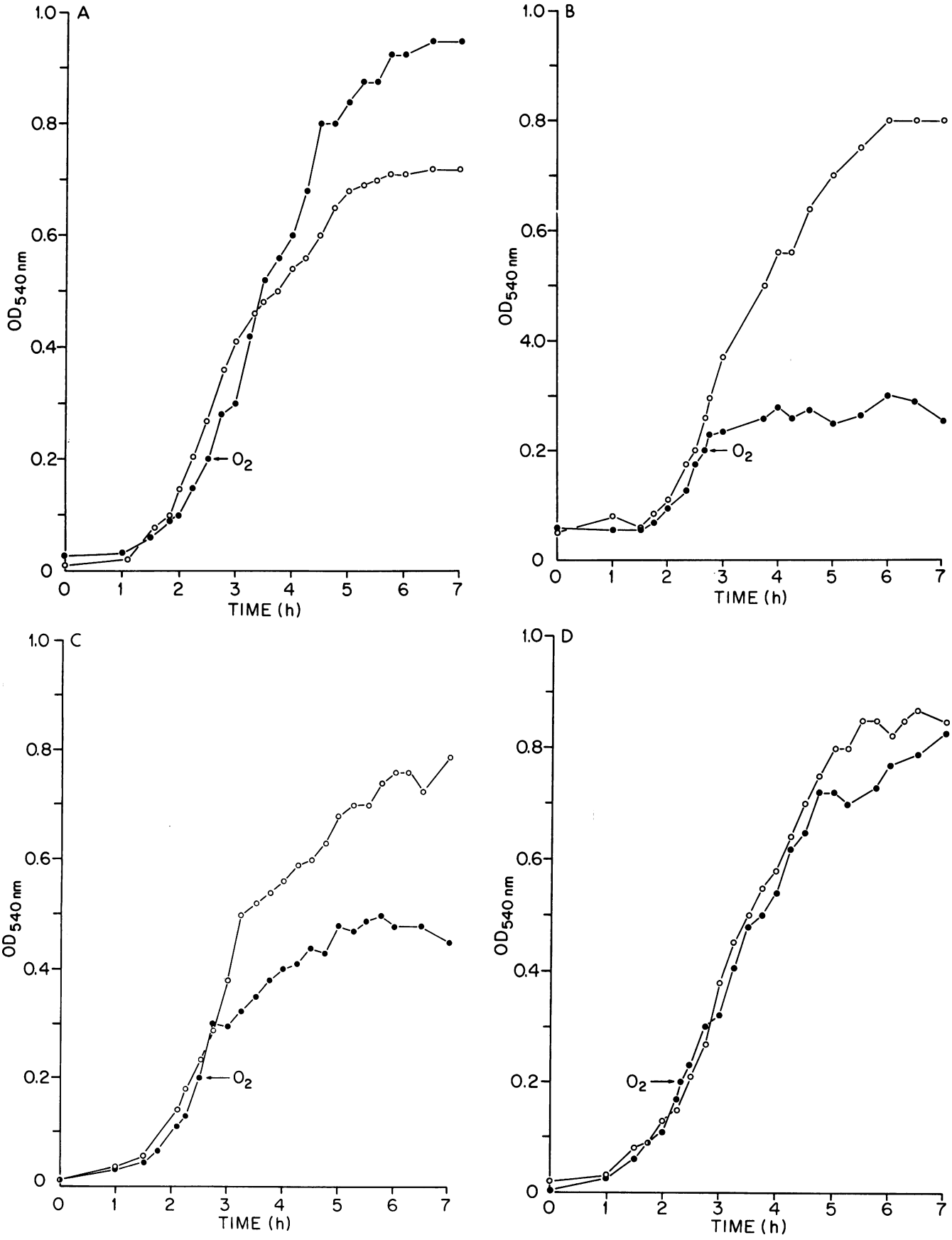
A
B
C
D
OD540 nm
TIME (h)
O2

TABLE 2. Genetic and enzymatic characteristics of the oxygen-sensitive mutants and the wild-type parent AB1157

Strain designation	Oxygen sensitivity class	Genetic location of mutation (map min)	Oxygen uptake (% removed/min per mg [dry wt])	Catalase (μmol of H_2O_2 removed/min per mg of protein)		Peroxidase (nmol of H_2O_2 consumed/min per mg of protein)		SOD (U/mg of protein)	
				Anaerobic	Oxygen exposed	Anaerobic	Oxygen exposed	Anaerobic (FeSOD)	Oxygen exposed (FeSOD + MnSOD)
AB1157			6.3	2.0	11.2	1.0	65.0	3.7	14.5
Oxy^s-2	I	8–13.5	ND[a]	ND	ND	ND	0.2	6.5	9.4
Oxy^s-3	I	8–13.5	1.5	ND	ND	0.04	0.2	1.2	2.9
Oxy^s-6	I	8–13.5	1.2	ND	ND	ND	1.7	4.5	3.7
Oxy^s-14	I	8–13.5	0.53	ND	ND	ND	ND	5.6	2.3
Oxy^s-17	I	8–13.5	2.1	ND	ND	ND	0.1	3.6	8.0
Oxy^s-4	I	77–83	0.64	ND	ND	ND	ND	3.0	0.2
Oxy^s-16	I	88–91.5	0.78	ND	ND	ND	0.9	3.8	0.5
Oxy^s-9	I	83–84.5	ND	5.5	34.5	32.3	334.2	2.2	13.0
Oxy^s-10	I	83–84.5	0.73	4.4	26.4	4.7	340.7	3.3	15.2
Oxy^s-12	I	88–91.5	0.60	0.48	1.6	ND	16.1	4.2	1.8
Oxy^s-1	II	77–83	1.6	3.9	20.5	1.8	517.9	4.1	15.3
Oxy^s-5	II	42–47	3.4	3.0	26.9	1.2	488.5	4.7	22.2
Oxy^s-7	II	83–84.5	3.2	4.9	30.3	ND	231.0	1.5	27.1
Oxy^s-11	III	47–51.5	8.3	2.4	11.0	ND	105.1	4.3	10.7
Oxy^s-13	III	47–51.5	5.0	1.5	7.8	ND	50.5	3.8	15.5

[a] ND, Not detectable by method used.

type parent (AB1157, OD_{540} = 1.0; Oxy^s-2, OD_{540} = 0.2; Oxy^s-9, OD_{540} = 0.45). Class I mutants stopped growing and dividing immediately after exposure of anaerobic cultures to either air or O_2 (Fig. 1B). Class II mutants, under these same conditions, continued to grow but at reduced rates and to a reduced final OD_{540} (Fig. 1C). The OD_{540} increase of class III mutants was unaffected by the introduction of air or oxygen (Fig. 1D), but microscopic observations of the cultures revealed that long nonseptate filaments were formed. These observations on liquid cultures correlated well with those made on microcolony development.

Genetic mapping and enzyme assays. With the 17 Hfr strains (Table 1), the mutations in the 15 oxygen-sensitive mutants were mapped to six chromosomal regions. The results of genetic mapping and biochemical assays are shown in Table 2.

Class I mutants. Of the 10 class I mutants, the mutations in 5 mutants mapped to 8 to 13.5 min. An F-prime strain covering the 6- to 12.5-min region was able to complement the mutation in each of these five Oxy^s mutants. Transduction mapping results for Oxy^s-6, Oxy^s-14, and Oxy^s-17 (Table 3) indicated that each of the mutations mapped to 8.4 min. Each of the mutants, Oxy^s-2, Oxy^s-3, Oxy^s-6, Oxy^s-14, and Oxy^s-17, had low oxygen uptake capacity, no detectable catalase activity, and low levels of peroxidase. The FeSOD level for each of these mutants was comparable to the anaerobically grown wild-type parent. After exposure to oxygen, total SOD (FeSOD plus MnSOD) activity of the mutants did not increase to the same extent as in the parent. In mutants Oxy^s-2, Oxy^s-3, and Oxy^s-17, total SOD activity increased slightly, but total SOD activity in Oxy^s-6 and Oxy^s-14 may have decreased slightly.

Two class I mutants, Oxy^s-4 and Oxy^s-16, had low oxygen uptake capacity, low catalase, low peroxidase, and SOD activity that decreased slightly upon exposure to oxygen. The extract of Oxy^s-16 fluoresced red when exposed to long-wave UV light. This indicated that a porphyrin precursor accumulated, possibly as a result of a genetic block in *hemE*, mapping at 90 min (33). A mutation in *hemE* could result in the accumulation of uroporphyrinogens I and III (33). The fluorescent compound accumulated by Oxy^s-16 has been tentatively identified as uroporphyrin on the basis of partitioning within the various extraction phases specified by Sasarman et al. (33). Calculations based on the absorbance of the Soret peak indicated that anaerobically grown Oxy^s-16 accumulated 1.955 nmol of porphyrin per g (dry weight), while the wild-type parent AB1157 accumulated no detectable porphyrins.

The three remaining class I mutants, Oxy^s-9, Oxy^s-10, and Oxy^s-12, mapped to 83 to 84.5, 83 to 84.5, and 88 to 91.5 min, respectively. In these mutants, the anaerobic (uninduced) catalase levels were similar to that of the wild-type parent. After exposure to oxygen, the catalase levels of AB1157 (wild type), Oxy^s-9, and Oxy^s-10 were induced approximately sixfold by exposure to oxygen. The catalase level of Oxy^s-12 was induced approximately threefold by exposure to oxygen. Anaerobic peroxidase levels in Oxy^s-9 were apparently higher than in the wild type; however, these

FIG. 1. Growth curves of the wild-type parent and class I, II, and III mutants. The effect of oxygen on liquid cultures was determined. Two cultures of each mutant were grown anaerobically to an OD_{540} of 0.2 (arrow). Then, one culture was bubbled with 100% oxygen for the remainder of the growth period (●). The other culture remained in 100% nitrogen (○). (A) Growth of the wild-type parent. (B) Growth of a class I mutant, Oxy^s-3. The class I mutants do not grow after exposure to oxygen. (C) Growth of a class II mutant, Oxy^s-5. The class II mutants are able to grow, but at a reduced rate and to a lower final titer, after oxygen exposure. (D) Growth of a class III mutant. Class III mutants filament in response to oxygen. If air, instead of 100% oxygen, is bubbled through the cultures, the results are the same.

TABLE 3. Mapping by P1 *vir* transduction

Mutant	Selected marker[a]	No. of Tet^r colonies	No. of Oxy^r colonies	Cotransduction frequency (%)	Location of gene for Oxy^r (min)[b]
Oxy^s-6	*aroL*::Tn*10*	298	111	37.2	8.39
	tsx::Tn*10*	67	6	8.96	8.3
Oxy^s-14	*aroL*::Tn*10*	230	79	34.3	8.35
	tsx::Tn*10*	18	2	11.1	8.36
Oxy^s-17	*aroL*::Tn*10*	247	91	36.8	8.38
	tsx::Tn*10*	84	12	14.29	8.45

[a] P1 *vir* lysates of JP3123 (*aroL*::Tn*10*) and χ2844 (*tsx*::Tn*10*) were used to transduce Oxy^s mutants to tetracycline resistance (Tet^r). Tet^r colonies were scored for the ability to grow aerobically (Oxy^r).

[b] The map distance from *aroL* (8.95 min) or *tsx* (9.4 min) to the gene for Oxy^r is calculated by the formula of Wu (43), where the length of the P1 phage is taken to be 2 min (4).The location of the gene for Oxy^r is calculated by subtracting the map distance from the genetic locations of the Tn*10* insertions.

levels were only induced 10-fold after exposure of the cells to oxygen as compared to a 65-fold induction for AB1157 and a 70-fold induction for Oxy^s-10. Peroxidase levels for Oxy^s-12 were induced by exposure to oxygen but the extent of induction could not be determined because of the low anaerobic levels. FeSOD levels for Oxy^s-9, Oxy^s-10, and Oxy^s-12 were similar to that of wild-type. Except for Oxy^s-12, these levels were induced to wild-type levels by exposure to oxygen. The reason for the decrease in total SOD levels for Oxy^s-12 has not yet been determined. In sum, the levels of catalase, peroxidase, and SOD for Oxy^s-9 and Oxy^s-10 were inducible to the same extent as for the wild-type parent, AB1157. The levels of catalase and peroxidase for Oxy^s-12 were inducible, but total SOD activity was not. Each of the mutants, Oxy^s-9, Oxy^s-10, and Oxy^s-12, had greatly diminished oxygen uptake capacities when compared with the wild-type parent, indicating possible respiratory deficiencies. Pyridine hemochrome spectra indicate the presence of cytochromes *b* and a_1 in anaerobically grown AB1157, Oxy^s-9, and Oxy^s-10. No pyridine hemochromes were detectable in Oxy^s-12 (data not shown). The relative amounts of type *b* cytochromes for AB1157, Oxy^s-9, Oxy^s-10, and Oxy^s-12, calculated from the absorption spectra, were 0.17, 0.15, 0.20, and 0.0 nmol/mg of protein, respectively. To rule out the possibility that the low respiratory activity of Oxy^s-12 was the result of a lack of quinones, menaquinone, ubiquinone, or *p*-hydroxybenzoate was added to NYG medium. These additions did not stimulate Oxy^s-12 to grow aerobically.

Class II mutants. The mutations in the class II isolates Oxy^s-1, Oxy^s-5, and Oxy^s-7 mapped to three separate chromosomal regions. Each of these mutants expressed oxygen uptake capacities that were slightly less than that of the wild-type parent. The catalase levels for each of these mutants before and after induction by oxygen were similar to that of the wild-type parent. The anaerobic levels of peroxidase for Oxy^s-1, Oxy^s-5, and Oxy^s-7 were similar to the wild-type level. Exposure to oxygen resulted in a several-hundredfold increase in peroxidase levels, which is much larger than the 65-fold increase observed for AB1157 (wild type). The significance of this large induction is not clear. The FeSOD levels of Oxy^s-1, Oxy^s-5, and Oxy^s-7 were similar to that of the wild-type parent. The total SOD levels of Oxy^s-1 and Oxy^s-5 were induced 3.7- and 4.7-fold, respectively, which compared favorably to a 4-fold induction in AB1157 (wild type) total SOD levels. The total SOD levels for Oxy^s-7 were induced to a greater extent than in the wild-type parent.

Class III mutants. The mutations in the class III mutants Oxy^s-11 and Oxy^s-13 mapped to the same chromosomal region. These mutants had wild-type oxygen uptake capacity and wild-type levels of catalase and SOD activity. The catalase and SOD enzyme levels were induced to the wild-type levels by exposure to oxygen. Peroxidase levels were induced by exposure to oxygen, most likely to a slightly greater extent than in the wild-type parent.

DISCUSSION

Our experiments show that mutations effecting oxygen sensitivity map to several chromosomal locations.

The mutations in 5 of the 10 highly oxygen-sensitive class I mutants, Oxy^s-2, Oxy^s-3, Oxy^s-6, Oxy^s-14, and Oxy^s-17, map to a single genetic region, 8 to 13.5 min. In all five mutants, there has been a simultaneous loss of several enzyme activities and a loss of ability to induce SOD to wild-type levels. In three of these, Oxy^s-6, Oxy^s-14, and Oxy^s-17, the mutations map to 8.4 min. This suggests that a single genetic locus at 8.4 min may coordinately regulate the expression of respiratory enzymes, catalase, peroxidase, and SOD in response to oxygen. The ability of an F-prime plasmid to complement the mutations in *trans* suggests that a diffusable factor may regulate these enzymes which are thought to protect cells from the toxic effects of oxygen. There is recent literature suggesting that the presence or absence of oxygen can have major effects on the simultaneous induction of genes (3, 37, 41) and proteins (35, 36).

The mutations in the class I mutants Oxy^s-4 and Oxy^s-16 map to separate chromosomal regions. Both Oxy^s-4 and Oxy^s-16 have low levels of oxygen uptake, catalase, peroxidase, and SOD. No previously mapped genes could be identified in the 77- to 83-min region that would account for the phenotype of Oxy^s-4. The mutation in Oxy^s-16 may be in the *hemE* gene. Mutants with a genetic block in this gene accumulate uroporphyrinogen (33), a porphyrin precursor required for synthesis of catalase and respiratory enzymes. A mutation in *hemE* could affect the function of several enzymes involved in oxygen metabolism, including catalase and cytochromes, resulting in oxygen sensitivity.

The remaining three class I mutants, Oxy^s-9, Oxy^s-10, and Oxy^s-12, have wild-type or slightly reduced levels of catalase, peroxidase, and SOD. However, these mutants have greatly diminished levels of oxygen uptake, suggesting that respiratory enzyme deficiencies may be responsible for their inability to grow in the presence of atmospheric oxygen. The decreased respiratory activity for Oxy^s-9 and Oxy^s-10 was probably not due to a lack of cytochromes, but may have resulted from a mutation affecting other portions of the respiratory chain. In the same region to which Oxy^s-9 and Oxy^s-10 map, the genes for the *E. coli* Ca^{2+}, Mg^{2+}-dependent adenosine triphosphatase, *unc* (12), are located (Fig. 2). A mutation in this operon could produce a loss of electron transport chain-coupled ATP synthesis capability in the presence of oxygen. The *unc* mutants are characterized by an inability to grow aerobically on succinate and poorly on glucose (12). A mutation in *unc* could conceivably result in the oxygen-sensitive phenotypes of Oxy^s-9 and Oxy^s-10. Pyridine hemochrome spectra suggest a lack, or low levels, of cytochromes for Oxy^s-12. Oxy^s-12 maps to a region containing four genes that may explain its phenotype (Fig. 2). Menaquinone is part of the *E. coli* respiratory chain and

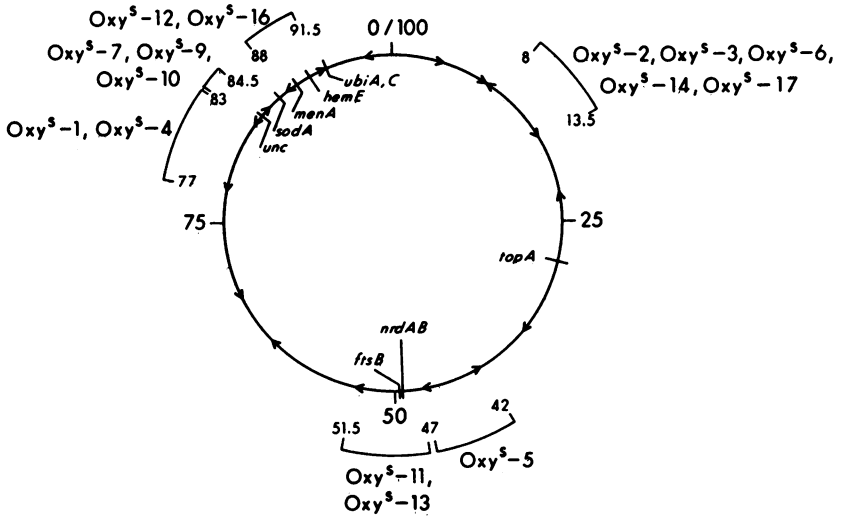

FIG. 2. Correlation of previously mapped genes with the genetic regions to which mutations effecting oxygen sensitivity map. The Oxy^s mutants map to six chromosomal regions. Within five of these regions, previously mapped genes are present that, when mutated, may very well give rise to oxygen sensitivity. Outside of these regions, other genes are also indicated. The arrowheads represent the origins of transfer of the Hfr strains listed in Table 1.

is synthesized, in part, by the *menA* gene product (21). In addition, *ubiA* and *ubiC* map just outside the 88- to 91.5-min region (4). Ubiquinone-deficient mutants are known to grow poorly aerobically but normally anaerobically (10). However, neither the addition of menaquinone nor that of *p*-hydroxybenzoate or ubiquinone could stimulate the aerobic growth of Oxy^s-12. The gene *hemE* also maps to this region (33). Although Oxy^s-12 does not accumulate extraordinary amounts of porphyrins, a mutation in *hemE* that reduces the levels of available porphyrins could account for the lack of detectable cytochromes and for the low levels of catalase activity found for Oxy^s-12.

Our observations suggest that Oxy^s-9, Oxy^s-10, Oxy^s-12, and Oxy^s-16 may be oxygen sensitive due to mutations in genes involved in respiration. By removing oxygen during the reduction to water at the cell membrane, respiratory enzymes may protect internal cellular components from oxygen toxicity.

In the class II mutants, Oxy^s-1, Oxy^s-5, and Oxy^s-7, oxygen sensitivity is expressed as a reduced growth rate and a lower final titer than that of wild type after exposure of liquid cultures to oxygen. No enzyme deficiencies were apparent that could account for the inability of the class II mutants to form colonies in the presence of oxygen (Table 2). We could not locate any reference to previously mapped genes in the particular genetic regions that, when mutated, could be expressed as a class II phenotype. To explain the class II phenotype, a model has been proposed in our laboratory. Perhaps, in the class II oxygen-sensitive mutants, a factor required for cell growth can no longer be produced in the presence of oxygen. When the cells encounter oxygen, production of the factor ceases. Once the cells run out of the factor (in a period equivalent to one or two cell divisions), cell growth stops.

The two class III mutants, Oxy^s-11 and Oxy^s-13, form nonseptate filaments in response to oxygen. Both of these mutants map to a single chromosomal region, 47 to 51.5 min (Fig. 2). The mutations may be alleles of a filamentation gene that has already been identified in this genetic region, *ftsB* (23, 38). The *ftsB* gene has recently been proposed to be an allele of *nrdB* (23, 38). The *nrdB* gene encodes a subunit of ribonucleotide reductase (39).

Two genes have been indicated in Fig. 2 that are not within the genetic regions containing the mutations of our oxygen-sensitive mutants. Genes *sodA* (40), encoding MnSOD, and *topA* (4), encoding topoisomerase I, both map outside of these genetic regions. The inability of most of the oxygen-sensitive mutants to induce MnSOD to wild-type levels is not due to a mutation in the structural gene, *sodA*. Mutations to oxygen intolerance in *S. typhimurium* were found to map near the *topA* gene (44). A lack of topoisomerase I activity was correlated with the inability to grow in the presence of oxygen (44). It is interesting to note that none of the oxygen-sensitive mutants we isolated map to a region containing the *topA* or *sodA* gene.

Our results demonstrate the variety of mutations that can result in oxygen sensitivity. Mutations in at least six chromosomal regions can effect sensitivity to oxygen. We have inferred the basis of some of these mutations from the genetic and biochemical data. Mutations in genes involved in respiration, cell division, or regulation of protective enzymes may affect the ability of cells to cope with oxygen and its toxic effects. The identification of these protective mechanisms may lead to an understanding of mechanisms of oxygen toxicity and of the way in which oxygen and oxygen radicals are involved in mutagenesis.

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