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Oxygen Sensitivity of an *Escherichia coli* Mutant

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Genetic evidence indicates that Oxy^s-6, an oxygen-sensitive mutant of *Escherichia coli* AB1157, is defective in the region of the *hemB* locus. Oxy^s-6 is capable of growth under aerobic conditions only if cultures are initiated at low-inoculum levels. Aerobic liquid cultures are limited to a cell density of 10^7 cells per ml by the accumulation of a metabolically produced, low-molecular-weight, heat-stable material in complex organic media. Both Oxy^s-6 and AB1157 cells produce the material, but only aerobic cultures of the mutant are inhibited by it. The material is produced by both intact cells and cell extracts in complex media. This reaction also occurs when the amino acid L-lysine is substituted for complex media.

Although it is generally agreed that enzymes such as catalase, peroxidase, and superoxide dismutase influence oxygen sensitivity, the exact mechanisms involved continue to be subjects for investigation. In addition, the possibility exists that other enzymes and nonenzymatic compounds are also important in determining an organism's ability to divide in the presence of oxygen. In order to explore the causes for oxygen sensitivity in bacteria, we isolated a group of 15 oxygen-sensitive mutants of *Escherichia coli* (5). These mutants were selected for their ability to form colonies on agar when incubated under anaerobic, but not aerobic, conditions. Preliminary characterization of the mutants suggested that at least six genetic loci and a variety of phenotypes were involved.

In this study, the mechanism responsible for oxygen sensitivity is explored and evidence is presented that one of the isolates, Oxy^s-6, has a mutation in the region of the *hemB* locus. The molecular structure and some of the functions of this locus have been established by other investigators (4, 8, 9, 13). It is known that *hemB* controls the synthesis of 5-aminolevulinate dehydratase, an enzyme required for the dimerization of 5-aminolevulinic acid to form the monopyrolle, porphobilinogen. Porphobilinogen is then converted, in a series of enzymatic steps, to heme. Heme is an important part of the active groups of catalase, peroxidase, and the cytochromes. Oxy^s-6 has been shown to be deficient in the activities associated with these heme-containing proteins, and therefore one might anticipate that its oxygen sensitivity would be related to internal concentrations of peroxides and related free radicals. However, we have found that the inability of this mutant to divide aerobically is a consequence of a low-molecular-weight material introduced to the external medium by the bacterium itself. This material appears to be a product of lysine metabolism.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* K-12 AB1157 (F$^-$ *thr leu pro his arg thi* Str^r) was obtained from E. A. Adelberg, Yale University. AB1157 and its oxygen-sensitive mutant, Oxy^s-6, have been maintained on refrigerated nutrient agar and in frozen nutrient broth suspensions for several years. Oxy^s-6-3.1 is a recombinant organism derived from Oxy^s-6 by electroporation as described below. *E. coli* MV1190 Δ(*lac-proAB*) *thi supE* Δ(*srl-recA*)*306*::Tn*10* (Tet^r) [F′ *traD36 proAB*$^+$ *lacI*^{q}ZΔ*M15*] (BioRad) and XL1-Blue *recA endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔ*M15* Tn*10*(Tet^r)] were used as hosts for plasmid constructions to localize the Oxy^s-6 mutation.

In most experiments, AB1157 and its derivatives were grown at 37°C in a nutrient broth consisting of 8 g of nutrient broth (Difco), 20 g of yeast extract, 6 g of sodium chloride, and 4 g of glucose (all in 1 liter of demineralized water) at pH 6.3 to 6.5. The unusually high yeast extract concentration and somewhat lower than usual pH were found to improve the reproducibility of final titers achieved by Oxy^s-6 under aerobic conditions. In some of the later experiments, 30 g of Casamino Acids (Difco) per liter of demineralized water at pH 6.3 to 6.5 was used as the medium. Growth of the organisms in this medium was equivalent to that in the nutrient broth described above. When anaerobic conditions were required, Oxyrase (Oxyrase, Inc., Ashland, Ohio) was added (0.1 ml/10 ml in a test tube [inner diameter, 23 mm]) just prior to inoculation, or the medium was purged continuously with a mixture consisting of 95% high-purity nitrogen and 5% CO_2 (1, 2).

In many experiments involving extended incubation periods, streptomycin sulfate (Sigma) was added to liquid medium to a final concentration of 50 mg/ml. AB1157, Oxy^s-6, and Oxy^s-6-3.1 are unaffected by streptomycin, even at 200 mg/ml. The numbers of CFU were determined under anaerobic conditions. In most experiments, pour plates containing Oxyrase were prepared as previously described (2) and incubated at 37°C for 24 h. In some experiments, pour plates without Oxyrase were incubated in anaerobic jars that were evacuated to −20 lb/in^2 and then filled with a mixture of 95% high-purity nitrogen and 5% CO_2. Evacuation and filling were performed three times before the jars were incubated. The two techniques produced equivalent numbers of CFU.

Enzymes. Restriction enzymes and T4 DNA ligase were obtained from Bethesda Research Laboratories and were used with the buffers and protocols supplied by the manufacturer.

Identification of recombinant λ phage which rescue Oxy^s-6. A number of recombinant λ (EMBL4) phages containing various regions of the *E. coli* genome were obtained from Y. Kohara, Nagoya University, Nagoya, Japan (7). These phages were tested for their ability to enable Oxy^s-6 to grow

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aerobically by coinfecting Oxy^s-6 with the recombinant phage and wild-type λ as described by Masters (11). DNA was prepared from phage which rescued Oxy^s-6 (10). Various fragments of this DNA were subcloned into pBS(+) (Stratagene) in order to localize the Oxy^s-6 mutation.

Electroporation of *E. coli*. To test their ability to complement Oxy^s-6, recombinant plasmids bearing various fragments of *E. coli* DNA were electroporated into Oxy^s-6 by following the protocol of Dower et al. (3).

Conditioned medium. Conditioned medium was prepared by inoculating fresh medium with Oxy^s-6 cells at a concentration of 1×10^5 to 3×10^5 cells per ml. The inoculum cells were obtained from a culture grown anaerobically for 16 to 18 h. The inoculated medium was incubated at 37°C with vigorous shaking under aerobic conditions for 9 to 16 h. At the end of this incubation, the medium was only slightly turbid and its pH remained 6.3 to 6.5. The medium was filter sterilized and stored at 4°C.

Dialysis of conditioned medium. Dialysis was performed by placing 10 ml of fresh medium inside a dialysis bag (Spectrophor; Fisher Scientific, Inc.) and 250 ml of conditioned medium outside the bag. After 18 to 24 h at 4°C with stirring, the contents of the bag were filter sterilized, inoculated with Oxy^s-6, and incubated. Initial and final titers were determined as described below under "Simplified assay."

Simplified assay. In preliminary experiments, conditioned media were assayed for the presence of inhibitor by inoculating the media and then titering repeatedly until the cultures reached a maximum cell density. In order to facilitate the experiments, a simplified version of this experiment, described below, was developed.

Media to be assayed for the presence of inhibitory materials were inoculated at approximately 2×10^5 cells per ml with Oxy^s-6 and incubated aerobically at 37°C until the maximum titer was attained (between 9 and 24 h). The numbers of CFU were determined at the beginning and end of the incubation period. The medium was considered conditioned if the bacterial titer increased by threefold or less during the incubation period. In fresh medium, the titer increased by 50- to 100-fold during the same incubation period.

If conditioned medium was diluted by as much as 20% with fresh medium, it retained its ability to inhibit growth completely. Further dilution permitted limited growth, the extent of which was approximately proportional to the dilution factor.

Preparation of cell extracts. Bacteria were grown under anaerobic conditions in 4-liter volumes of medium contained in Cell Production Roller Vessels (Bellco). Inoculum densities were approximately 2×10^5/ml, and incubation was at 37°C for 16 to 20 h. Cells were harvested by centrifugation (20 min, 3,000 × *g*) and washed once in 0.03 M phosphate buffer at pH 6.8. They were then resuspended in the same buffer (1.8 ml of buffer per g [wet weight]) and passed four times through a cold French press at approximately 20,000 lb/in^2. Cell debris was removed by centrifugation (30 min, 17,300 × *g*) at 4°C. The dry weight of the extract was determined, and small aliquots (0.5 ml) were stored at −70°C.

Conversion of lysine to inhibitor by extracts. A solution of L-lysine (A grade, Cal Biochem) in 0.02 M phosphate buffer (pH 6.3) was brought to 37°C in a water bath or incubator. In most experiments, the L-lysine concentration was 1.5 mg/ml. To this solution, cell extract was added (final concentration, approximately 0.4 mg (dry wt) of extract per ml). At intervals, 5-ml samples were removed and the reaction was quenched by boiling for at least 20 min. Each sample was then cooled, and 15 mg of Casamino Acids (Difco) was added. The pH was readjusted to 6.3 if necessary. This procedure produced a 3% Casamino Acids medium. This medium was filter sterilized, and the presence of an inhibitor derived from lysine was determined by the simplified assay.

Thin-layer chromatography. Five-microliter samples were applied in 1-μl increments with drying between applications, onto Eastman cellulose sheets (20 by 20 cm). The ascending chromatography solvent system consisted of 30 parts of 30% ammonium hydroxide and 70 parts of 100% *n*-propanol (Fisher Scientific). The solvent fronts were allowed to flow 15 cm, at which time the sheets were removed from the chromatography jar, air dried, and sprayed with a 3% ninhydrin acetone solution. They were then air dried and baked in an oven at 120°C for 3 to 5 min.

RESULTS

Localization of the Oxy^s-6 mutation. Earlier work had located Oxy^s-6 in the interval between *lacZ* and *proC* (5). Several recombinant λ phages which contain *E. coli* DNA from this region were obtained and tested for their ability to rescue Oxy^s-6 (7). Two phages, λ140 and λ141, were able to restore the ability of Oxy^s-6 to grow aerobically. Fragments of the *E. coli* DNA contained in λ140 were subcloned into pBS(+) and the region which rescued Oxy^s-6 was localized to the *proC* proximal 3.1 kb of this DNA (pOR3 [Fig. 1]). Dividing this interval at the unique *Hin*dIII site located within the *hemB* locus yielded two subclones (pOR5 and pOR6) which no longer complemented Oxy^s-6.

The *hemB* gene, which encodes δ-aminolevulinic dehydratase, has been cloned and sequenced (8, 9). A clone of *hemB*, pJL2 (kindly supplied by S. Cosloy of The City College of The City University of New York), when introduced into Oxy^s-6, restored its ability to grow aerobically. These genetic observations, taken collectively, suggest that the mutation in Oxy^s-6 involves the *hemB* locus. These observations do not, however, prove that the mutation is entirely restricted to this locus.

Properties of a recombinant clone. One of the recombinant clones produced by electroporation with pOR3 (Oxy^s-6-3.1) was characterized more completely. In addition to its ability to form large colonies on the surface of agar under aerobic conditions, it could also grow to more than 10^9 cells per ml in aerobic liquid cultures. Its rate of growth was slightly less than that of AB1157. As was the case for AB1157, Oxy^s-6-3.1 was not inhibited by conditioned medium or the inhibitor made from lysine (discussed below).

The levels of catalase, peroxidase, and superoxide dismutase in Oxy^s-6-3.1 were similar to those observed in AB1157 and considerably higher than those in Oxy^s-6 (Table 1). The small amount of superoxide dismutase found in Oxy^s-6 (Table 1) is the iron-containing form of the enzyme; the other organisms contain, in addition, the manganese and hybrid forms (4a).

Table 1 also establishes that δ-aminolevulinic acid dehydratase, the product of the *hemB* locus, is not present in Oxy^s-6. Table 1 shows that as previously observed, the level of the enzyme is elevated in the recombinant organism (9). The δ-aminolevulinic acid dehydratase assays were done using sonicates from anaerobically grown cells.

Phenotypic observations. When Oxy^s-6 was inoculated into fresh nutrient broth at a cell density of approximately 2×10^7 cells per ml or greater, little division took place even after many hours of aerobic incubation. This confirmed earlier

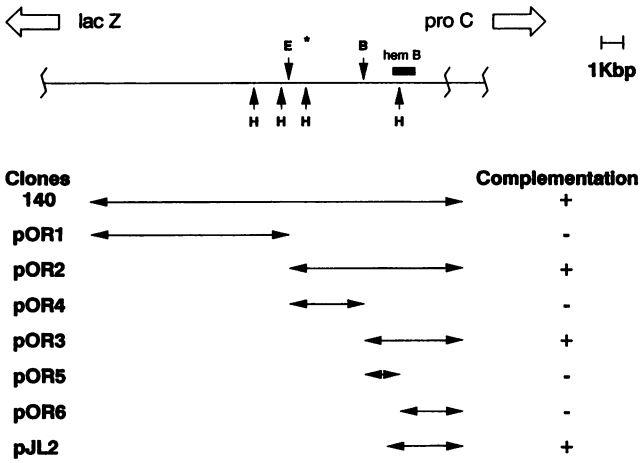

FIG. 1. Physical map of the *hemB* region of the *E. coli* genome and Oxy^s-6 complementation properties of various subclones. The upper part of the figure is a restriction map of this region with arrows to the nearby markers *lacZ* and *proC* for orientation. The restriction enzyme cleavage sites used were *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H). The *Eco*RI site marked with an asterisk is about 26 kbp from the *Eco*RI site in the 3′ end of the *lacZ* gene. Each line under the map identifies a DNA segment contained in one of the various subclones. The ability of various subclones to complement Oxy^s-6 is indicated in the column on the right. pOR1 and pOR2 are *Eco*RI fragments beginning from an *Eco*RI site in the λ cloning vector (EMBL4) and ending at an *Eco*RI site internal to the segment of *E. coli* DNA. pOR3 and pOR6 have a *Bam*HI site that presumably resulted from the combination of a *Sau*3A site, from the *Sau*3A partial digest of the *E. coli* DNA used in the construction of the original library and the *Bam*HI site in the EMBL4 cloning vector. DNA subclones were introduced into Oxy^s-6 by electroporation, and cells that could form colonies under aerobic conditions were selected.

observations (5). If, however, the inoculum level was below 10^7 cells per ml, division took place until a titer of approximately 10^7 cells per ml was reached. This titer is approximately 100-fold lower than that reached by the parental organism, AB1157. Over a wide range of inoculum densities, the number of divisions that occurred in Oxy^s-6 was always sufficient to bring the final titer to 1×10^7 to 3×10^7 cells per ml (Table 2). At this titer, 10-ml cultures in 125-ml Erlenmeyer flasks were only very slightly turbid. Anaerobic cultures of Oxy^s-6, initiated at any inoculum level, reached a

TABLE 1. Enzymatic properties of AB1157, Oxy^s-6, and Oxy^s-6-3.1

Strain	Enzyme content of cell extracts (U/mg of protein)			
	5-Aminolevulinic acid dehydratase[a]	Catalase[b]	Peroxidase (10^2)[b]	Superoxide dismutase[b]
AB1157	0.023	20.85	10.89	36.1
Oxy^s-6	0.000	0.08	0	0.319
Oxy^s-6-3.1	0.230	14.02	7.77	34.6

[a] Determined by the method of Sassa (14).
[b] These observations were kindly provided by Hosni M. Hassan, Department of Microbiology and Food Science, North Carolina State University, Raleigh, North Carolina. They were made using previously published methods (15). *ortho*-Dianisidine was used as the oxidizable substrate in the peroxidase assay.

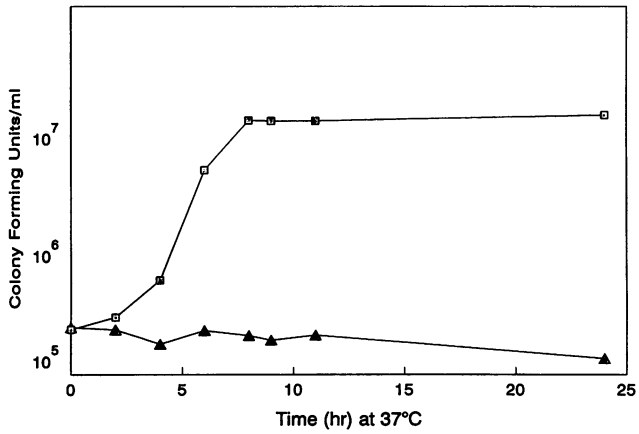

FIG. 2. Growth of Oxy^s-6 under aerobic conditions. Symbols: ▨, growth in fresh medium; ◬, growth in conditioned medium (see Materials and Methods for preparation of conditioned medium).

final titer of approximately 2×10^8 cells per ml and were very turbid.

In the course of our effort to explain why the growth of Oxy^s-6 is limited to approximately 10^7 cells per ml under aerobic conditions, experiments were performed in which Oxy^s-6 cells from an overnight anaerobic culture were inoculated into two samples of nutrient broth. One sample consists of fresh nutrient broth, the other was conditioned by prior aerobic incubation with Oxy^s-6 cells (see Materials and Methods). In fresh medium, the organism grew to approximately 10^7 cells per ml, but in conditioned medium, little or no aerobic growth occurred (Fig. 2). Conditioned medium had no inhibitory effect on the aerobic or anaerobic growth of AB1157, the parent of Oxy^s-6.

Although conditioned medium was usually produced under aerobic conditions as described in Materials and Methods, it could also be produced by Oxy^s-6 when growing anaerobically. Table 3 shows that a culture of Oxy^s-6 growing under anaerobic conditions rapidly conditions the medium so that it becomes inhibitory when subsequently assayed aerobically. Table 3 also demonstrates that although the anaerobically growing cells condition the medium in which they are growing, this inhibitory property does not interfere with growth of the cells to a high titer (2.2×10^8) under anaerobic conditions.

Properties of conditioned medium. In order to determine whether the inhibitory effect of conditioned medium is due to an agent introduced by the prior growth of Oxy^s-6 or the depletion of an essential growth factor, experiments were done in which conditioned medium was freeze-dried and

TABLE 2. Final titers of Oxy^s-6 cultures initiated at several inoculum levels[a]

Inoculum density	Aerobic final titer	Anaerobic final titer
1.7×10^7	3.6×10^7	1.7×10^8
1.8×10^6	2.2×10^7	1.9×10^8
1.3×10^5	2.1×10^7	1.4×10^8
1.7×10^4	2.1×10^7	1.9×10^8

[a] The inoculum came from an anaerobic culture of Oxy^s-6. The inoculum cells were washed once in nutrient broth and then diluted appropriately. Final titers were determined at 24 h.

TABLE 3. Production of conditioned medium under anaerobic conditions[a]

Incubation time (h)	Titer in anaerobic incubation mixture	Simplified assay	
		Initial titer	Final titer
0	2.1×10^7	2.2×10^5	2.3×10^7
1	3.9×10^7	2.2×10^5	1.9×10^5
1.5	5.5×10^7	2.2×10^5	2.2×10^5
2.0	9.8×10^7	2.2×10^5	1.1×10^5
2.5	1.6×10^8	2.2×10^5	1.1×10^5
3.0	2.2×10^8	2.2×10^5	1.5×10^5

[a] Anaerobically grown Oxy^s-6 cells were diluted into fresh nutrient broth that was sparged with a mixture of 95% N_2 and 5% CO_2. At the indicated times, the titer of the incubation mixture was determiend and a sample was filter sterilized for use in the simplified assay.

TABLE 4. Production of inhibitor from nutrient broth by cell extracts[a]

Treatment	Simplified assay	
	Initial titer	Final titer
Control (fresh medium)	1.8×10^5	1.4×10^7
Oxy^s-6 extract + nutrient broth	1.4×10^5	1.2×10^5
AB1157 extract + nutrient broth	1.4×10^5	3.8×10^6
Oxy^s-6 extract + 3% Casamino acids	1.6×10^5	1.0×10^5
Oxy^s-6 extract + water extract of unhydrolyzed casein	1.6×10^5	1.8×10^7

[a] Extracts were reacted with the substrates for 2 h at 37°C. The final concentration of extracts was 0.4 mg (dry wt)/ml of reaction mix. The reactions were terminated by boiling.

then reconstituted by the addition of fresh medium. Such reconstituted medium remained completely inhibitory. Fresh medium similarly reconstituted permitted typical aerobic growth. Conditioned medium survived autoclaving at 120°C for 0.5 h. The inhibitory agent(s) in conditioned medium passed through 1,000- but not through 100-molecular-weight-cutoff dialysis tubing.

Various low-molecular-weight metabolic compounds were added to fresh medium to determine whether they inhibited growth of Oxy^s-6. Sodium acetate (10 mM), sodium lactate (10 mM), sodium formate (10 mM), ethanol (20 mM), 5-aminolevulinic acid (10 mM), and hydrogen peroxide (0.1 mM) failed to mimic the inhibitory agent(s) present in conditioned medium. Because Oxy^s-6 is known to be catalase negative, the hypothesis that metabolically produced hydrogen peroxide might be the inhibitory agent was particularly attractive, and therefore several experiments were performed in which catalase was added either during the conditioning period or to medium that had already been conditioned. None of these experiments supported the suggestion that hydrogen peroxide was the inhibitory agent. In addition, a sample of fresh medium was exposed to 50,000 R of 250 kV X-rays, a procedure known to produce free radicals and hydrogen peroxide. The irradiated medium had no inhibitory properties.

Oxy^s-6 can condition medium rapidly and completely even in the absence of appreciable cell division. This occurred when cells were suspended in fresh medium at a concentration of 10^7 cells per ml or greater. In such experiments, the conditioning of medium was obvious after a 2-h incubation and essentially complete after 3 h. Similar experiments performed with *E. coli* AB1157 showed that the parental organism also accumulated the inhibitor(s) but at a somewhat slower rate.

Production of inhibitor by cell extracts. Table 4 shows that cell extracts from anaerobically grown Oxy^s-6 and AB1157 will condition nutrient broth. With the incubation period used, the AB1157 extract was less effective than that of Oxy^s-6. In experiments (not shown) in which longer incubations were carried out, the AB1157 extracts did produce completely conditioned medium. Table 4 also shows that a less complex medium made of Casamino Acids (Difco) can be conditioned by an Oxy^s-6 extract. An extract prepared by mixing 3 g of unhydrolyzed casein (Difco) with 100 ml of demineralized water could not be conditioned.

The results shown in Table 4 suggested that the precursor for the inhibitor might be one of the free amino acids present in acid-hydrolyzed casein (Casamino Acids; Difco). Therefore, experiments were conducted in which various combinations of reagent amino acids were treated with Oxy^s-6 extract, freeze-dried, and then resuspended in media which were used in the simplified assay. It was shown that cell extracts could produce a material that inhibits the aerobic growth of Oxy^s-6 only on mixtures containing lysine. It was eventually determined that lysine could be converted to inhibitor in the absence of any other amino acids. Table 5 shows that an Oxy^s-6 extract rapidly converts lysine to an inhibitory product. An AB1157 extract also carried out this reaction but, as was the case with intact cells, did so at a slower rate. The production of the inhibitor(s) in the lysine-extract incubation medium was accompanied by the appearance of a ninhydrin-positive lysine product(s) that could be detected on thin-layer chromatographic plates (Fig. 3). The intensity of the product spot is qualitatively correlated with the biological effectiveness of the inhibitor (compare Table 5 and Fig. 3.)

Table 6 demonstrates that the inhibitor(s) made from lysine had properties consistent with the presence of the inhibitor(s) in conditioned medium. The product from L-lysine inhibited the aerobic growth of Oxy^s-6. It had little or no effect on anaerobic growth. It had no significant effect on the aerobic or anaerobic growth of AB1157. Both inhibitors pass readily through a 1,000-molecular-weight-cutoff membrane filter.

DISCUSSION

The primary purpose of this contribution is to describe the physiological and biochemical basis for oxygen sensitivity in *E. coli* Oxy^s-6. However, the genetic experiments performed

TABLE 5. Production of inhibitor from lysine by cell extracts

Treatment and reaction time (min)	Simplified assay	
	Initial titer	Final titer
Oxy^s-6 extract + lysine		
0	2.8×10^5	3.6×10^7
10	2.8×10^5	2.3×10^5
20	2.8×10^5	2.5×10^5
30	2.8×10^5	2.4×10^5
60	2.8×10^5	2.5×10^5
AB1157 extract + lysine		
0	2.8×10^5	3.9×10^7
10	2.8×10^5	1.3×10^7
20	2.8×10^5	2.8×10^6
30	2.8×10^5	1.6×10^6
60	2.8×10^5	2.2×10^5

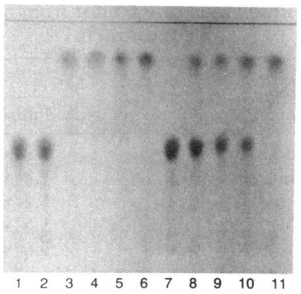

FIG. 3. Thin-layer chromotography of product made from lysine by Oxy^s-6 and AB1157 extracts. L-Lysine (lane 1) and extracts of Oxy^s-6 (lanes 2 to 6) and AB1157 (lanes 7 to 11) at 0 (lanes 2 and 7), 10 (lanes 3 and 8), 20 (lanes 4 and 9), 30 (lanes 5 and 10), and 60 (lanes 6 and 11) min.

allow us to suggest that the mutational basis for oxygen sensitivity may reside at or near the *hemB* locus. The single most critical observation suggesting that the *hemB* locus is involved is the fact that if the plasmid pOR3, which complements the mutation in Oxy^s-6, is cleaved at the unique *Hin*dIII site within the *hemB* gene, neither of the two subclones derived (pOR5 and pOR6) complements this mutation (Fig. 1). The suggestion is supported by the fact that Oxy^s-6 shares several phenotypic properties with known *hemB* mutants. Oxy^s-6, like other *hemB* mutants, lacks 5-aminolevulinic dehydratase, the direct product of the *hemB* locus (Table 1). Also, like other *hemB* mutants, it is deficient in catalase and peroxidase.

Certain features of Oxy^s-6 suggest that its mutation may extend beyond the *hemB* locus. The organism has a diminished content of superoxide dismutase, is unusually oxygen sensitive, and is remarkably stable. Although Oxy^s-6 has been used in many experiments for at least 5 years, we have never observed any spontaneous reversions to oxygen tolerance. This last observation suggests that a genetic deletion may be responsible for the complex phenotype of Oxy^s-6. Until more-detailed information becomes available, we are assuming that the phenotype of Oxy^s-6 is due to a genetic alteration, possibly a deletion, in the 3.1-kb region that includes the *hemB* locus.

Although the first description of Oxy^s-6 suggested that it could not divide in liquid medium under aerobic conditions, the current experiments establish that this is true only when the initial inoculum is 10^7 cells per ml or greater. At inoculum levels lower than 10^7/ml, growth does occur until a concentration of approximately 10^7 cells per ml is attained. This cell density is 100 to 200 times less than that attained by the parental organism, AB1157. Under anaerobic conditions, both organisms grow to densities greater than 10^8 cells per ml, regardless of inoculum concentration. The almost immediate cessation of division previously observed (5) when relatively high-titer cultures of Oxy^s-6 were transferred from anaerobic to aerobic conditions can now be explained. It was almost certainly a response to an extracellular inhibitor that was produced during the anaerobic growth period but only became effective when cultures were transferred to aerobic conditions.

TABLE 6. Inhibitory properties of the product made from L-lysine[a]

Organism inoculated	Final titer	
	Control medium	Lysine product medium
Oxy^s-6		
Aerobic	2.2×10^7	1.7×10^5
Anaerobic	3.3×10^8	2.0×10^8
AB1157		
Aerobic	4.0×10^9	3.5×10^9
Anaerobic	4.0×10^8	3.8×10^8

[a] All cultures were inoculated at an initial cell density of approximately 2×10^5 cells per ml and then grown for 16 h. The control medium was nutrient broth. The lysine product was obtained after 30 min of reaction with Oxy^s-6 extract and then fortified with dry nutrient broth ingredients (see Materials and Methods).

In this work, we present evidence that the oxygen sensitivity of Oxy^s-6 is due to a metabolically produced inhibitor found in the external medium. Experiments in which the conditioned medium was freeze-dried and then reconstituted with fresh medium made it unlikely that inhibition reflected a loss of a medium component. The conclusion that conditioned media were not lacking essential nutrients was further supported by the observation that AB1157 grew very well in such media, and Oxy^s-6 itself had no difficulty in growing in such media under anaerobic conditions. The observation that cells and cell extracts can make an inhibitor from lysine further supports the conclusion that the phenomenon originally observed was due to the production of a new compound(s) from medium components rather than the depletion of an essential nutrient. Both of the complex media used in our experiments have significant concentrations of free L-lysine. It is interesting to note that materials mutagenic for bacteria can be formed by heating mixtures of glucose and lysine (6). However, these materials are probably not closely related to the inhibitor discussed here because they require the participation of glucose and are sensitive to catalase. Neither the inhibitor described here or the process by which it is made is sensitive to catalase.

At the present time, little is known about the factors in cell extracts responsible for converting lysine to inhibitor or about the chemical nature of the inhibitor(s). We are assuming that the inhibitor(s) made when cells are incubated with complex media and the inhibitor(s) made when cell extracts are incubated with L-lysine are the same material.

When efforts were begun to explain why Oxy^s-6 had difficulty growing under aerobic conditions, we did not anticipate that an extracellular, metabolically produced factor would be involved. To our knowledge, no such factor has previously been suggested to account for the failure of an organism to grow under aerobic conditions. It should be noted that this inhibitor is bacteriostatic rather than bacteriocidal. Aerobically inhibited Oxy^s-6 cells do divide and form colonies when diluted and plated under anaerobic conditions, as was done in many experiments described here. Likewise, liquid aerobic cultures of Oxy^s-6 that have reached stasis will resume replication if returned to an anoxic environment.

We have observed that Oxy^s-6, in addition to being sensitive to the inhibitor, seems to accumulate this material more rapidly than the parental culture, AB1157. One attractive hypothesis that will be tested in future experiments is the possibility that AB1157 converts the inhibitor to an innocuous compound, whereas Oxy^s-6 lacks this ability. If this conversion were to involve a heme-containing enzyme, a role for the *hemB* locus would be plausible. For example, it is known that cadaverine, a well-known derivative from lysine and a possible candidate for the inhibitor of aerobic growth, can be converted to an amine aldehyde by an

oxidative deaminase (12). During this reaction, hydrogen peroxide is generated. This inhibits the continued activity of the deaminase. AB1157 contains the heme enzymes, catalase, and peroxidase and would therefore be able to remove the peroxide and propagate the deamination. Oxy^s-6 could not do this, and cadaverine would be expected to accumulate. In future work, we will attempt to determine the chemical nature of the inhibitor and the presumed enzyme system that produces it.

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