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Isolation of the *Escherichia coli* iron superoxide dismutase gene: Evidence that intracellular superoxide concentration does not regulate oxygen-dependent synthesis of the manganese superoxide dismutase

(stopped-flow spectrophotometric assay)

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ABSTRACT **A mixed-sequence synthetic oligodeoxynucleotide probe was used to identify clones within the *Escherichia coli* genomic library of Clarke and Carbon having an extrachromosomal copy of iron superoxide dismutase. Plasmids pLC13-47 and pLC18-11 were shown to contain the structural gene of the iron superoxide dismutase and to overproduce this protein under conditions of chloramphenicol amplification of plasmid copy number. The activities of both manganese and iron proteins were measured in extracts of host cells and plasmid-bearing cells grown over a wide range of oxygenation. The results confirm previous demonstrations that the manganese protein is repressed under anaerobic conditions and induced in the presence of oxygen. Induction of the manganese protein with increasing oxygenation was quantitatively similar in cells differing ≈7-fold in iron superoxide dismutase, suggesting that the intracellular concentration of superoxide might not be responsible for regulating synthesis of the manganese-containing superoxide dismutase.**

Escherichia coli cells are able to synthesize two distinct types of superoxide dismutase (EC 1.15.1.1); one contains iron, the other, manganese (see refs. 1 and 2 for review). Both proteins are found in the cytosol (3) and both have approximately equal specific superoxide dismutase activity (4, 5). The iron protein has been reported to be constitutive under most growth conditions, including anaerobiosis (6), whereas the manganese protein is induced in the presence of oxygen (6, 7–9). Hassan and Fridovich (10, 11) have shown that a variety of redox dyes that promote formation of O_2^- can cause a dramatic induction of the manganese superoxide dismutase while having little effect on the iron protein. These authors concluded that superoxide rather than oxygen was directly or indirectly responsible for induction of the manganese superoxide dismutase (10). Assuming a reciprocal relation between O_2^- and superoxide dismutase concentrations, it follows that cells containing a high concentration of active iron superoxide dismutase would be expected to suppress induction of the manganese protein. In this communication, we describe the isolation of *E. coli* strains bearing the iron superoxide dismutase gene on a plasmid and capable of attaining a high intracellular level of this protein. The variation of the two superoxide dismutases in one of these strains and appropriate control strains as a function of oxygenation has been examined, and the results form the basis of this report.

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Protein Sequence	N – (24)	Glu	– Tyr	– His	– Tyr	– Gly
Message	5′	GA(A/G)	– UA(U/C)	– CA(U/C)	– UA(U/C)	– GG( )
Probe	3′	CT(T/C)	– AT(A/G)	– GT(A/G)	– AT(G)	– CC( )

FIG. 1. Design of the mixed-sequence DNA probe molecule for the iron superoxide dismutase gene of *E. coli*.

MATERIALS AND METHODS

Bacterial Strains, Genomic Library, Media, and Sources of Materials. The Clarke–Carbon (12) plasmid library and the parent strain, *E. coli* JA200 (*thr leuB∇ trpE recA thi ara lacY galK galT xyl mtl* λ *supE44*), were obtained from F. C. Neidhardt, The University of Michigan, Ann Arbor, MI.

Colicin was prepared and used according to the method of Spudich *et al.* (13). Phage T4 polynucleotide kinase was obtained from P-L Biochemicals, adenosine 5′-[γ-^{32}P]triphosphate (triethylammonium salt) (specific activity 3000 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham, and nitrocellulose type BA-85 was obtained from Schleicher & Schuell. All other chemicals were of the highest commercial quality.

General Recombinant DNA Methodology. Unless otherwise noted the procedures described by Maniatis *et al.* (14) were followed.

Design and Preparation of the Probe. An 8-fold degenerate probe, 14 bases in length (Fig. 1), was synthesized by the triester method (15) and purified essentially according to the detailed description of Cohn *et al.* (16). The design of this probe was based on the NH_2-terminal amino acid sequence published by Steinman and Hill (17) (Fig. 1). The 8-fold rather than 16-fold degeneracy of the synthetic 14-mer was based on early hybridization experiments with a 16-fold degenerate probe in which C was inadvertently substituted for G at position 12 (3′ → 5′). This probe was found to have a very low affinity for denatured *E. coli* DNA, leading us to believe that G was the complementary base at position 12.

The sequence of the synthetic probe was confirmed by Robert Landick, using the Maxam–Gilbert method (R. Landick, D. Maguire, and L. C. Lutter, personal communication).

Manipulations with the Probe. Electrophoresis of probe labeled with [^{32}P]ATP after the final chromatography purification step suggested that the 14-mer represented ≈90% of the

Abbreviations: FeSD, iron superoxide dismutase; MnSD, manganese superoxide dismutase.
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material present and that further purification was not required for hybridization experiments. In these experiments 0.13 μg of probe was labeled with [^{32}P]ATP as described by Maniatis *et al.* (14) and diluted into 10× Denhardt's solution (14)/6× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate). The probe was hybridized to DNA that had been immobilized on a nitrocellulose filter. After 3 hr at room temperature (21°C) the nitrocellulose filter was rinsed for 1 hr at room temperature in several changes of 6× NaCl/Cit then treated for 2 min at 35°C in 6× NaCl/Cit. It was then exposed to Kodak X-Omat AR film for 18–20 hr at −70°C.

Electrophoresis. Cell extracts and purified protein were subjected to polyacrylamide gel electrophoresis under nondenaturing conditions (18). The gels were stained for protein with Serva blue G and for superoxide dismutase activity by the method of Beauchamp and Fridovich (19).

Stopped-Flow Measurement of Superoxide Dismutase Activity. The instrumentation and examples of the numerical analysis were described previously by Bull *et al.* (20). The stopped-flow system was used to generate a solution of O_2^- having an initial concentration ≈0.1 mM in the presence or absence of a cell extract. Data collection consists of observing the decay of the O_2^- by its absorbance at 260 nm; the initial A_{260} was ≤1. Data analysis consists of fitting the decay curve to an appropriate differential equation. Thus, in the presence of a mixture of iron and manganese superoxide dismutases (FeSD and MnSD) the theoretical expression describing disappearance of O_2^- is

$$\frac{d[O_2^-]}{dt} = k_1[O_2^-] + k_2[O_2^-]^2 + \frac{k_{Fe}[FeSD][O_2^-]}{[O_2^-] + K_m^{Fe}} + \frac{k_{Mn}[MnSD][O_2^-]}{[O_2^-] + K_m^{Mn}}, \quad [1]$$

in which ks are rate constants and K_ms are Michaelis constants. Analysis of decay curves in the presence of pure proteins has allowed us to determine all the constants in this expression. Under the conditions used here (see legend to Fig. 3) $K_m^{Fe} >> [O_2^-]_0$ so that iron dismutase should display first-order kinetics with $k_{Fe}/K_m^{Fe} = 1.0 \times 10^8\ M^{-1}\ s^{-1}$. We assume a value of $k_{Mn}/K_m^{Mn} = 2.2 \times 10^8\ M^{-1}\ s^{-1}$ for the *E. coli* manganese protein.§ The measured K_m^{Mn} for the *E. coli* protein was 3.7×10^{-6} M, which allows us to calculate $k_{Mn} = 810\ s^{-1}$.

To analyze for the two superoxide dismutases in a crude cell extract, data were first taken in the absence of cell extract to obtain k_1 and k_2 under conditions of the experiment. We then examined the catalytic activity of an extract that had been treated with 8 mM H_2O_2 for approximately 10 min to destroy the iron protein (21). (Diluted extracts so treated had an additional absorbance at 260 nm of ≈0.1 unit due to the presence of H_2O_2. Since total protein in these dilutions is less than 0.1 mg/ml, there is insufficient catalase present to destroy the H_2O_2. Longer incubation times or further additions of H_2O_2 caused no further decrease of activity.) Analysis of these data gave [MnSD] by using the previously determined values of k_1, k_2, k_{Mn}, and K_m^{Mn}. Finally, the catalytic activity of the untreated extract (no peroxide) was measured and corrected for the effects of k_1, k_2, k_{Mn}, and K_m^{Mn}. With the above value for k_{Fe}/K_m^{Fe}, the remaining velocity yielded [FeSD]. Least-squares fitting to the above expression never involved finding more than two parameters from any one experiment. Control experiments involved examination of mixtures of purified *E. coli* iron dismutase and *Thermus thermophilus* manganese protein and addition of known concentrations of these pure proteins to the extracts; the expected results were obtained. On this evidence the method is valid. Its details will be reported elsewhere.

Superoxide dismutase levels are reported as nmol of bound metal ion per g of cellular protein. An extract containing 560 nmol of manganese and 80 nmol of iron protein per g of protein exhibited 68 units/mg of protein in the standard cytochrome *c*/xanthine oxidase assay system (22). Purified iron dismutase gave a value of 4800 units/mg (5).

Cell Growth. Cultures (50 ml) of *E. coli* cells were grown at 37°C on LB medium or LB medium plus glucose (56 mM). Colicin was included when cells contained the ColE1 plasmid. Cells were grown under nitrogen, in the presence of air, and under sparging with pure oxygen. A rotary shaker was used to suspend the cells and to provide vigorous aeration. Oxygenation of the culture medium was carried out by simultaneous shaking and sparging at a flow rate of 1 liter of $O_2\ hr^{-1}$ through coarse-grade bubblers. Cells were generally harvested by centrifugation at $OD_{600} \approx 0.6$.

Plasmid Amplification. Cells were grown aerobically to $OD_{600} \approx 0.9$, chloramphenicol (34 mg/ml in 95% ethanol) was added to a final concentration of 50 μg/ml, and the culture was incubated for 5 hr. At the end of this period the cells were washed to remove chloramphenicol, resuspended in fresh medium, and allowed to double in cell density [method adapted from Young *et al.* (23)] prior to harvesting.

Preparation of Cell Extracts. Cells were harvested by centrifugation and suspended in 5 ml of 0.2 M glycine, pH 9.6, with 0.1 mM diethylenetriaminepentaacetic acid, and sonicated for two 1-min periods with a Branson sonifier equipped with a standard microtip. The extracts were clarified by centrifugation at 20,000 rpm in a Sorvall SS-34 rotor for 10 min and the supernatant was assayed for superoxide dismutase activity in the stopped-flow apparatus. The pro-

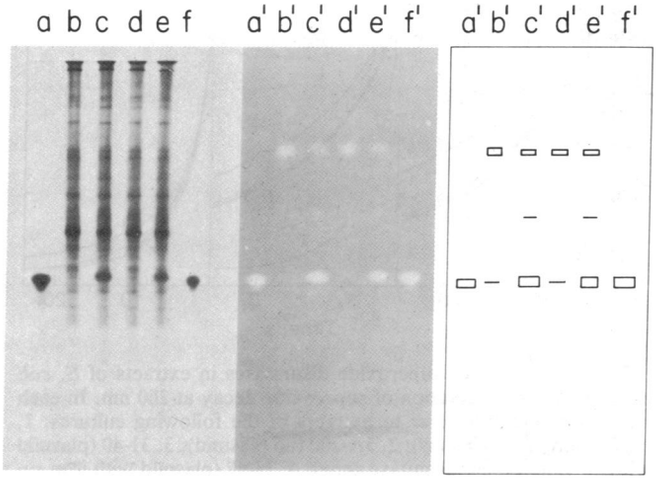

FIG. 2. Gel electrophoresis of *E. coli* cell extracts under nondenaturing conditions. (*Left* and *Center*) Lanes a, a', f, and f' contained approximately 0.11 nmol of Fe as Fe dismutase. Lanes b, c, d, and e and b', c', d', and e' contained 40 μg of protein extracted from strains JA-200, 13-47, 31-40, and 18-11, respectively. The leftmost gel was stained for protein with Serva blue G, and the center gel was stained for superoxide dismutase activity according to the method of Beauchamp and Fridovich (19). (*Right*) Schematic of the gel stained for superoxide dismutase activity. Cells were grown aerobically with plasmid amplification. The upper bands are due to the Mn protein; the lower bands are due to the Fe protein; and the weak intermediate bands are due to a small amount of hybrid material.

§There are complications in the assay, not fully described here, which do not affect the relative values of superoxide dismutase concentrations given in this communication. (*i*) The iron protein undergoes a partial inactivation during assay. (*ii*) Since purified manganese protein from *E. coli* was not available, we determined the value of $k_{Mn}/K_m^{Mn} = 2.2 \times 10^8\ M^{-1}\ s^{-1}$ for the *Thermus thermophilus* protein under our experimental conditions. This agrees with literature values obtained for this and several other manganese proteins, which are $2–4 \times 10^8\ M^{-1}\ s^{-1}$.

tein content of the clarified extracts was measured by the method of Lowry *et al.* (24).

RESULTS AND DISCUSSION

Approximately 1600 colonies from the Clarke–Carbon bank were screened by the colony transfer method (25). The plasmids were isolated from 22 of the most promising of these and were electrophoresed along with the plasmids isolated from 5 strains that showed very low affinity for the labeled probe. The DNA was blotted onto nitrocellulose and hybridized with the ^{32}P-labeled probe. Plasmids pLC13-47 and pLC18-11 (carried in strains 13-47 and 18-11, respectively) exhibited the highest affinity for the probe, and these were selected for further experiments.

The apparent positive clones 13-47 and 18-11, the negative clone 31-40, and the parent strain, JA-200, were grown and amplified with chloramphenicol. Equal amounts of protein from each of these strains were subjected to polyacrylamide gel electrophoresis, and the gels were stained for both protein and superoxide dismutase activity (19) as shown in Fig. 2. The results strongly suggest overproduction of the iron protein by 13-47 and 18-11 but not by 31-40 or JA-200.

To test independently whether iron superoxide dismutase activity is correspondingly higher in 13-47 and 18-11, the superoxide dismutase activity of cell extracts was measured. Fig. 3 shows decay curves of superoxide in the presence of equal concentrations of protein in extracts of 13-47, 31-40 (negative), and JA-200. It is immediately apparent that the specific superoxide dismutase activity of 13-47 is very much greater than that of 31-40 or JA-200 (Fig. 3*A*).¶ When the extracts were incubated with H_2O_2, most of the dismutase activity was lost and the activity remaining, due to the manganese protein, was approximately equal in all strains. The amounts of iron and manganese proteins in four different strains are summarized in Table 1.

We wished to determine whether the high levels of iron superoxide dismutase obtainable in these clones might suppress the induction of the manganese protein that occurs under conditions of strong oxygenation of the culture medium. For these experiments we chose strain 13-47 because it grew at rates closer to those of the parent strain, JA-200. The strain having pLC18-11 was found to grow at a somewhat lower rate than that of the plasmidless host, as was noted previously by Grogan and Cronan (26). Cells were grown in LB medium and LB medium plus glucose (in the absence of chloramphenicol) under conditions of oxygenation ranging from anaerobiosis to sparging with pure oxygen. Data for the two superoxide dismutases are reported in Table 2. Under

¶Experiments with maxicells confirm the presence of the structural gene of iron superoxide dismutase on plasmids pLC18-11 and pLC13-47 along with several other chromosomal proteins (V. Vaughn and F. C. Neidhardt, personal communication).

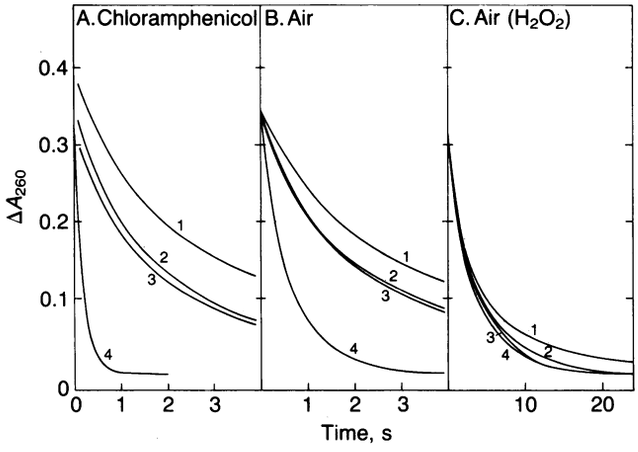

FIG. 3. Assay for superoxide dismutases in extracts of *E. coli* cells by direct observation of superoxide decay at 260 nm. In each panel, the numbers refer to extracts of the following cultures: 1, buffer only (no additions); 2, JA-200 (no plasmid); 3, 31-40 (plasmid without superoxide dismutase gene); 4, 13-47 (plasmid with iron superoxide dismutase gene). In *A*, cultures were grown aerobically with shaking and chloramphenicol treatment was used to amplify the plasmid copy number. Cultures assayed in *B* were grown aerobically and were not treated with chloramphenicol. *C* shows the effect of adding H_2O_2 to the samples used in *B* (note change in abscissa). This treatment inactivates the iron superoxide dismutase so that the presence of the manganese protein may be observed. Conditions after mixing: Protein concentration was 0.015 mg/ml in *A* and 0.020 mg/ml in *B* and *C*. The absorbance due to the extracts was between 0.5 and 0.6 for the 2-cm pathlength. The concentration of O_2^- was approximately 100 μM in 0.2 M glycine buffer at pH 9.6 containing 0.1 mM diethylenetriaminepentaacetic acid, 5% (vol/vol) dimethyl sulfoxide, and ovalbumin at 0.05 mg/ml.

Table 1. Effect of plasmids on the levels of superoxide dismutases in *E. coli* cells

	[FeSD]		[MnSD]	
Strain	nmol/g	Enhancement	nmol/g	Enhancement
JA-200	95	1	171	1
31-40	156	1.6	141	0.8
18-11	2076	22	195	1.1
13-47	3076	32	190	1.1

Amounts of superoxide dismutase are given in nmol of Fe or Mn per g of extracted protein. All cells were subjected to the chloramphenicol treatment to amplify plasmids.

Table 2. Effects of various growth conditions on the levels of superoxide dismutases in *E. coli* cells

Conditions and strain	Growth rate, hr^{-1}	[FeSD], nmol/g	[MnSD], nmol/g
	LB medium		
Anaerobic*			
Aerobic (shaking)	1.44 ± 0.13		
JA-200		91 ± 22 (3)	81 ± 15 (4)
31-40		100 (1)	130 (1)
13-47		746 ± 175 (6)	133 ± 10 (2)
O_2 sparge	1.06 ± 0.12		
JA-200		48 ± 18 (3)	495 ± 102 (3)
31-40		80 (1)	560 (1)
13-47		235 ± 74 (2)	539 ± 118 (2)
	LB + glucose medium		
Anaerobic	1.27 ± 0.19		
JA-200		50 ± 10 (3)	≈0
13-47		310 ± 113 (4)	≈0
Aerobic (shaking)	1.53 ± 0.12		
JA-200		32 ± 11 (2)	45 ± 13 (3)
13-47		357 ± 151 (3)	84 ± 43 (2)
O_2 sparge	1.15 ± 0.13		
JA-200		22 ± 9 (3)	261 ± 40 (3)
13-47		166 ± 40 (2)	380 ± 73 (2)

Cells were harvested at OD_{600} = 0.6 ± 0.05. Amount is expressed as nmol of superoxide dismutase-bound metal ion per g of cellular protein. All measurements are included, and the indicated uncertainty is the average deviation from the mean. The number of independent experiments is given in parentheses. Growth rates are averages for all strains grown under the particular conditions.

*Cells cultured anaerobically on LB medium had a very short log-linear growth period. As expected, no manganese protein could be detected. The activity of the iron protein, measured in one culture of JA-200 during this period, was 66 nmol of FeSD per g of cell protein, and this increased to 360 as the cells reached stationary phase. This behavior will be described in more detail elsewhere.

the specified conditions growth was log-linear and cells were harvested in logarithmic phase. Data are not shown for anaerobic cultures in LB medium because growth was not log-linear in the absence of glucose, and the level of the iron dismutase increased dramatically as the cells approached stationary phase (cf. footnote to Table 2).

There are several noteworthy features of these data: (*i*) Cells carrying pLC13-47 produce rather large amounts of the iron protein (as well as the other proteins expressed by this plasmid[¶]) without affecting their growth rate. (*ii*) As expected from previous work (6, 7–9), oxygen induced the manganese protein, and we observed this in all strains. (*iii*) An unexpected result was that the iron protein in cells containing pLC13-47 was somewhat repressed by increased oxygenation: ≈3-fold from air to oxygen in the LB medium and ≈2-fold from anaerobic to O_2 in the LB + glucose medium. To test whether the iron protein was inactivated by oxygen, we subjected several cultures to O_2 sparging in the presence of a static level of chloramphenicol and observed no decrease in iron protein levels after 1 hr. The significance of these changes is unclear, however, since we do not know if the plasmid copy number changes under the different growth conditions. Previous work (6) indicated that the *E. coli* iron protein was constitutive, and the small changes we see in the host strain may not be significant. (*iv*) Glucose repressed both superoxide dismutases. This is not a large effect, being ≈2-fold in all comparable experiments, and is consistent with previous results (27). (*v*) Finally, the level of the manganese protein was independent of the level of the iron protein. This is most striking in the data for aerobic growth, where a 7- to 10-fold difference in the concentration of iron protein had no effect on the level of the manganese protein.

The above results localize the iron superoxide dismutase gene on the Clarke–Carbon plasmids pLC13-47 and pLC18-11. Grogen and Cronan (26) have begun studies with pLC18-11, noting that it carries a gene or genes that affect the rate of growth of the host organism as well as a gene or genes responsible for cyclopropane fatty acid synthesis. These authors have located the latter at ≈36.5 min on the *E. coli* linkage map.[‖] Assuming no genetic rearrangements have occurred, it is thus reasonable to place the iron superoxide dismutase gene near 36.5 min on the linkage map. Recently, Touati (28) isolated the *E. coli* manganese superoxide dismutase gene on a cosmid and, using the method of λ phage cotransduction, located this gene near 87.5 min on the linkage map. Thus, the iron superoxide dismutase gene, here named *sodB*, and the manganese dismutase gene, *sodA* (28) are approximately 51 min apart on the *E. coli* chromosome (cf. ref. 29).

Earlier results of Gregory and Fridovich (7–9) and Hassan and Fridovich (6, 10, 11) showed that the manganese protein is powerfully induced either by mild hyperoxia (cf. also ref. 30) or by redox drugs that are reduced by cellular constituents and in turn react with oxygen to produce superoxide. These results were logically interpreted to mean that superoxide was responsible for the oxygen-dependent induction of the manganese protein. The results presented in Table 2, however, show that the induction of manganese protein by oxygenation is the same in *E. coli* cells having widely differing levels of iron protein. Since the two types of superoxide dismutases appear to be in the cytosol (3) and the intracellular concentration of O_2^- might be expected to be inversely proportional to the total concentration of superoxide dismutase, the present data indicate that superoxide itself may not be the inducer of the manganese protein.

[‖]Personal communication from D. W. Grogan and J. E. Cronan. These authors have sent us a culture of their strain 18-11, and we have confirmed its ability to overproduce the iron superoxide dismutase. Further, pLC18-11 and pLC13-47 have overlapping restriction fragments. Our DNA probe binds exclusively to the 4.9-kilobase *Bam*HI/*Eco*RI fragment (cf. ref. 26).

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