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Production of superoxide dismutases from *Proteus mirabilis* and *Proteus vulgaris*

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***Proteus mirabilis* and *Proteus vulgaris* expressed a combination of superoxide dismutase (Sod) activities, which was assigned to FeSod1, FeSod2 and MnSod for *P. mirabilis*, and FeSod, MnSod and CuZnSod for *P. vulgaris*. Production of the Sod proteins was dependent on the availability of iron, whether cells were grown under anaerobiosis or aerobiosis and growth phase. Nalidixic acid and chloramphenicol inhibited cell growth and the iron- and dioxygen-dependent production of Sod. These results support the involvement of metal ions and redox status in the production of *Proteus* Sods.**

Keywords: superoxide dismutase, *Proteus*, iron metabolism, pathogen

Introduction

Proteus mirabilis and *Proteus vulgaris* are Gram-negative bacteria typically associated with human urinary tract infections. They begin as 2 μm swimmer cells in a vegetative stage and upon contact with surfaces differentiate through an elongation process to 40–80 μm hyperflagellated swarmer cells in a swarm stage, which appears to be important for invasive colonization of human urothelial cells (Allison *et al.* 1992). Both rapid growth and swarming are dependent on adequate levels of nutrients, particularly iron. In response to the low iron environment of the urinary tract, *Proteus* elaborates virulence factors, including specific outer membrane proteins (Shand *et al.* 1985), hemolysins (Mobley *et al.* 1991) and amino acid deaminases (Drechsel *et al.* 1993), in a concerted effort to scavenge iron from red blood cells and other cellular stores.

The involvement of iron in virulence and the requirement for iron in swarmer cell differentiation (Jin & Wurray 1988), coupled with the observation that swarming was characterized by decreased respiratory activity (Armitage 1981), led us to examine *Proteus* for iron- and dioxygen-dependent superoxide dismutase (Sod) production. Bacteria possess a combination of FeSod, MnSod and CuZnSod. The CuZnSod is less commonly found, perhaps a result of evolutionary determinants or its sensitivity to temperature, pH and physical trauma during extract preparation (Benov & Fridovich 1994). Renewed interest in Sod has been motivated by reports that mutated, but otherwise active, CuZnSod appeared linked to amyotrophic lateral sclerosis (Gurnery *et al.* 1994) and that FeSod expression in *Campylobacter jejuni* has been implicated in bacterial invasion (Pesci *et al.* 1994). Most of our current understanding of Sod has come from the molecular studies dealing with the regulation of *Escherichia coli sod* genes by combination of the positive regulatory factors, which comprise superoxide response (*soxRS* and *soxQ*) loci, the negative regulatory factors, which include aerobic respiration control (*arcA*), ferric uptake regulation (*fur*), fumarate-nitrate reduction (*fnr*), DNA gyrase (*gyr*) and integration host factor loci (Niederhoffer *et al.* 1990, Hassan & Sun 1992, Wu & Weiss 1992, Compan & Touati 1993, Privalle *et al.* 1993), and the characterization of amino acid auxotrophy and membrane permeability of *sod* pseudo-revertants (Fee *et al.* 1988, Imlay & Fridovich 1992), although there have been selected reports from other microorganisms (see, e.g. Hassett *et al.* 1993, May & Dennis 1989). In the present work, we report on the characterization of Sod activity from *P. mirabilis* and *P. vulgaris*.

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Materials and methods

Strains, media and reagents

P. mirabilis 7002 and *P. vulgaris* 33420 were obtained from the ATCC (Rockville, MD). Cultures were grown in L broth or M9 minimal medium plus 0.2% glucose and 20 $\mu g\,ml^{-1}$ thiamine. Single colonies were maintained on non-swarming LSW^- agar as described by Belas *et al.* (1991). All reagents were of the highest commercial quality. Media components were obtained from Difco.

Siderophore assays

Siderophore production was evaluated by spreading stationary phase cultures of *P. mirabilis* 7002, *P. vulgaris* 33420 and *E. coli* strains W3110 (Fur^-) and ECN1 (Fur^-) (Foster *et al.* 1994), which were grown in iron-depleted M9 medium plus appropriate antibiotics, on chrome azurol S (CAS) universal assay medium (Schwyn & Nielands 1987) with the following modifications. Concentrated medium was prepared by mixing 1.5 g of Bactor agar, 2 ml 50-fold mineral salts (30 g KH_2PO_4, 5 g NaCl and 10 g NH_4Cl in 200 ml of H_2O), 3 g of piperazine-*N*,*N*-bis(2-ethanesulfonic acid) and 83 ml of H_2O. The pH was adjusted to below 6.8 with 5 M NaOH. The CAS Fe solution was prepared by mixing 1 ml 10 mM $FeCl_3$, 2 ml 10 mM hexadecyltrimethylammonium bromide and 6 ml of H_2O. The solutions were sterilized in an autoclave, allowed to partially cool and slowly mixed by pouring the CAS–Fe solution into the concentrated medium. If the resulting solution had a green color, five or six drops of concentrated HCl were added to generate a blue working stock, which was then poured into Petri dishes. The presence of enterobactin, aerobactin and hydroxamate-containing compounds was confirmed by previously described analytical techniques (Arnow 1937, Atkin & Nielands 1968, Gillam *et al.* 1981) on cultures grown in M9-based minimal medium in the presence, absence or restriction of iron (Foster *et al.* 1994).

Cell growth

Overnight cultures of individual *Proteus* strains were grown in L broth at 37°C with rapid shaking (200 r.p.m.) and were used to provide 1% inocula to L broth supplemented with 50 or 100 μM $FeSO_4$, 100 μM diethylenetriaminepentaacetic acid (DTPA), or 25 or 100 μM MV for aerobic cultures contained in nephelo flasks or anaerobic cultures contained in sealed, screw-topped culture flasks. Strains were incubated at 37°C with rapid shaking until they reached various phases of growth. Typically, late exponential phase (about 350 for aerobic and about 100 for anaerobic cultures as recorded with a Klett–Summerson colorimeter) was obtained in 3–9 h for the aerobic cultures and 8–12 h for the anaerobic cultures. These studies were performed three separate times.

Hydroxyurea, nalidixic acid, rifampicin or chloramphenicol were used to inhibit DNA replication, DNA transcription or RNA translation, in the presence and absence of iron (Moody & Hassan 1984). Cells were grown to stationary phase in L broth and used to inoculate fresh L broth. When a reading of 50–100 was measured with a Klett–Summerson colorimeter, a combination of inhibitors, $FeSO_4$ and DTPA was added to give a final concentration of 250 $\mu g\,ml^{-1}$ hydroxyurea, 250 $\mu g\,ml^{-1}$ nalidixic acid, 250 $\mu g\,ml^{-1}$ rifampicin, 250 $\mu g\,ml^{-1}$ chloramphenicol, 100 μM $FeSO_4$ or 100 μM DTPA and the cultures were incubated for 1.5–3 h at 37°C. These experiments were performed twice.

For aerobic induction of protein production, 3 ml of an aerobically grown L broth culture was used to inoculate 300 ml of fresh L broth contained in a 250 ml serum bottle. The bottle was sealed and incubated for 12–18 h, during which time the available dioxygen was depleted. This anaerobic culture was used to provide a 50 ml inoculum to 50 ml of L broth medium contained in a 500 ml culture flask, the total volume containing 100 μM $FeSO_4$ or DTPA in the absence or presence of 250 $\mu g\,ml^{-1}$ nalidixic acid or chloramphenicol. The cultures were incubated at 37°C for 1.5 h. This study was performed twice.

Preparation of cell extracts

All manipulations were carried out at 4°C. Cells were collected by centrifugation at 8000 *g*, washed and suspended in a pH 8 solution containing 50 mM Tris and 1 mM EDTA, ruptured by sonication for two intervals of 15 s with a VirSonic 300 (VirTris Company, Gardiner, NY) equipped with a standard microtip, and centrifuged at 12 700 *g* to remove cellular debris. Total protein concentrations were determined by the bicinchoninic acid method (Smith *et al.* 1985); bovine serum albumin served as the calibration standard.

Sod activity

The xanthine–xanthine oxidase-coupled reduction of cytochrome *c* assay as described by McCord & Fridovich (1969) was used to quantify total soluble Sod activity.

Electrophoresis

Cell extracts and purified *E. coli* FeSod and MnSod and bovine CuZnSod proteins (Sigma, St Louis, MO) were electrophoresed under non-denaturing discontinuous conditions on 10% polyacrylamide (30:0.8) resolving and 4% stacking gels (Hames 1990). The gels were stained for Sod, catalase or peroxidase activity by the method of Beauchamp & Fridovich (1971), Clare *et al.* (1984) and Misra & Fridovich (1977), respectively. The type of Sod was tentatively assigned based on specific chemical inhibition of achromatic zones; FeSod was assayed in the presence of 20 mM H_2O_2 with or without 2.5 to 5 mM KCN (to inhibit catalase and peroxidase) or 5 mM NaN_3 and CuZnSod was assayed in the presence of 5 mM KCN, incubation of samples was performed in a pH 8 solution containing 50 mM Tris and 1 mM EDTA at 37°C for 1 h prior to electrophoresis or by soaking the resolved polyacrylamide gels in the above solutions at room temperature for 1 h prior to Sod-specific staining. Intensities were quantified from 8-bit (256 gray

levels) images obtained with an Apple Color OneScanner and Ofoto software with analysis performed on a Macintosh Quadra 840AV computer using the public domain NIH Image program (written by Wayne Rasband at the US National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disks from NTIS, 5285 Port Royal Road, Springfield, VA 22161, part number PB93-504868). There was a variance of ±20 to 25% in the measured intensities. High-density, 600 d.p.i. output was obtained from a calibrated LaserWriter Select 360.

Results

Iron-dependent growth in rich medium

Proteus grew well in L broth independent of supplementation with 50 or 100 μM $FeSO_4$. However, when cultures were made iron-limited by supplementation with the iron chelator DTPA (100 μM), growth rate slowed but final cell density was unaffected. No evidence was found for the production of enterobactin, aerobactin or hydroxamate-containing ligands by pure cultures in low iron, defined minimal medium. After several days of incubation on CAS universal assay plates, we detected limited zones of yellow–orange colorization surrounding colonies of the *Proteus* strains.

Sod specific activity

Total soluble Sod specific activities were measured for anaerobic, aerobic and aerobic plus MV cultures that were grown to late exponential phase. We observed 5 U mg^{-1} for anaerobic DTPA-supplemented, 15 U mg^{-1} for anaerobic iron-supplemented, 20 U mg^{-1} for aerobic iron- and DTPA-supplemented, and 25 U mg^{-1} for aerobic MV-supplemented cultures. The variance in individual measurements was ±20%.

Sod type identification

So that we could make direct comparisons of relative Sod specific activities between individual electrophoretograms, the same amount (20 μg) of protein from late exponential phase cultures was loaded into each lane on the polyacrylamide gels illustrated in Figure 1. When appropriate, active Sod production was determined from scanned 8-bit images of carefully prepared gels; there was a variance of ±20 to 25% in the scanned intensities. There was no detected interference from catalase or peroxidase (results not shown). We referred to the individual bands as M1, M2 and M3 for *P. mirabilis* 7002 and V1, V2 and V3 for *P. vulgaris* 33420, signifying the most mobile band to the least mobile band of Sod activity, respectively. Fresh extracts yielded three sharp achromatic zones on polyacrylamide gels (indicated by the labels in Figure 1), while repeated freeze–thaw cycles yielded additional minor bands (proteolysis products migrated ahead of M1 or V1 and hybrids midway between M1 and M3 or V1 and V2). We found that bands M1 and M2 of *P. mirabilis* 7002 and bands V1 of *P. vulgaris* 33420 were inactivated in the presence of 20 mM H_2O_2 and 2.5 mM KCN, which was consistent with FeSod1 (M1 and V1) and FeSod2 (M2). Bands M3 and V2 were resistant to chemical inactivation, which was consistent with MnSod. Band V3 was completely inhibited with 5 mM KCN, which was consistent with CuZnSod.

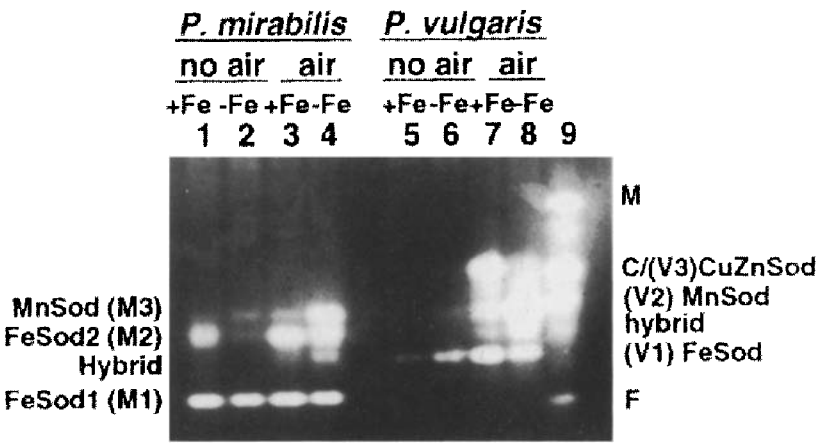

Figure 1. Visualization of Sod activity from cellular extracts of *P. mirabilis* 7002 and *P. vulgaris* 33420. Lanes 1–4 and 5–8 contained crude cellular extracts from growing cultures of *P. mirabilis* 7002 and *P. vulgaris* 33420, respectively. Extracts in lanes 1, 2, 5 and 6 were from cells grown anaerobically; extracts in lanes 3, 4, 7 and 8 were from cells grown aerobically. Extracts in lanes 1, 3, 5 and 7 were from cells grown in the presence of 100 μM $FeSO_4$, extracts in lanes 2, 4, 6 and 8 were from cells grown in the presence of 100 μM DTPA. Lane 9 contained 1 μg each of purified *E. coli* MnSod (M) and FeSod (F) and bovine CuZnSod (C). The data are representative of three independent trials.

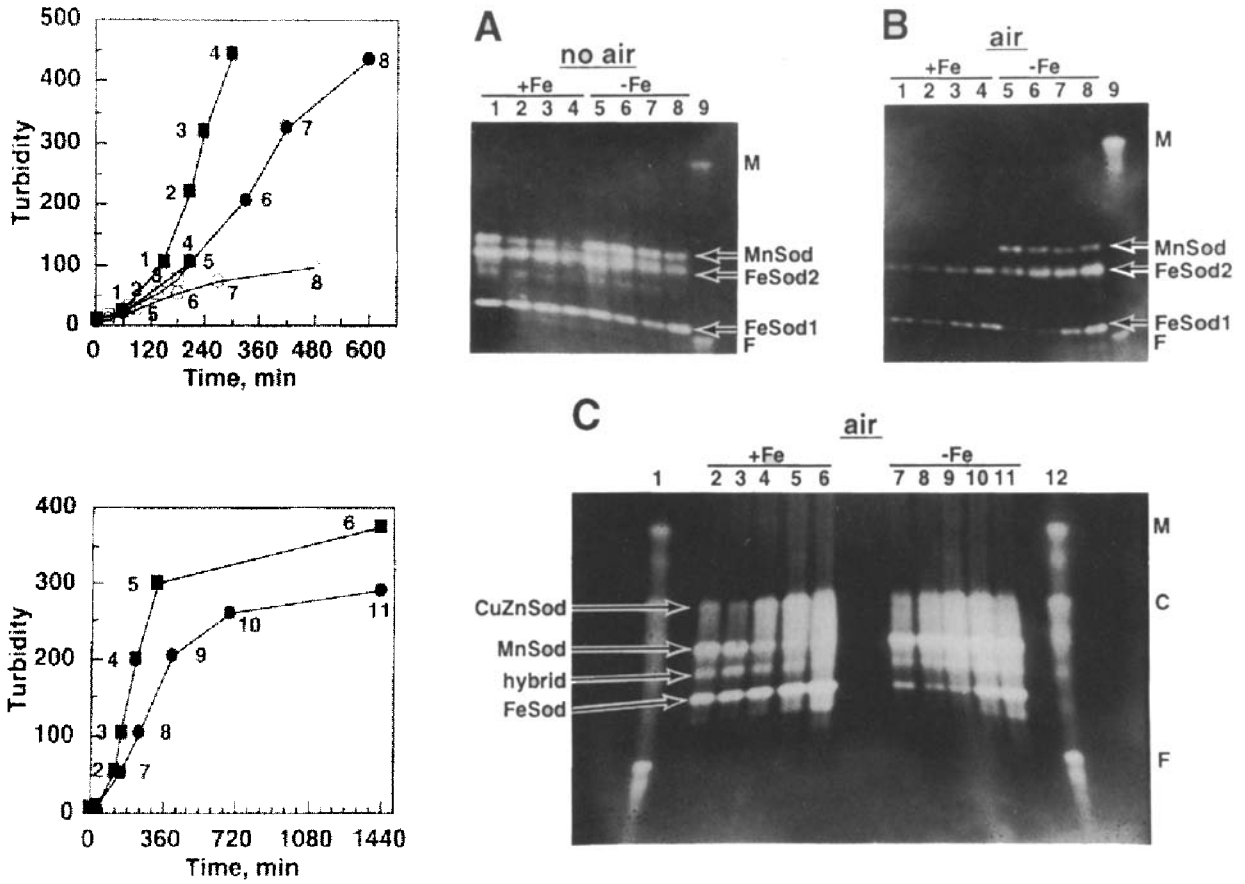

Figure 2. Growth phase production of Sod from *P. mirabilis* (**A** and **B**) and *P. vulgaris* (**C**). Samples for PAGE analysis were taken at time points indicated by corresponding lane numbers on the individual growth curves. Open and closed symbols represent anaerobic and aerobic cultures, respectively. Squares and circles signify growth in the presence of 100 μM $FeSO_4$ and 100 μM DTPA, respectively. For *P. mirabilis* 7002, extracts were prepared from cells grown anaerobically (**A**) or aerobically (**B**) in the presence of 100 μM $FeSO_4$ (lanes 1–4) or 100 μM DTPA (lanes 5–8). Lane 9 contained 1 μg each of purified *E. coli* MnSod (M) and FeSod (F). For *P. vulgaris* 33420, extracts were prepared from cells grown aerobically (**C**) in the presence of 100 μM $FeSO_4$ (lanes 2–6) or 100 μM DTPA (lanes 7–11). Lanes 1 and 12 contained 1 μg each of purified *E. coli* MnSod (M) and FeSod (F) and bovine CuZnSod (C). The data are representative of two independent trials.

Sod production in P. mirabilis *7002*

A FeSod1 (M1) was expressed both under anaerobiosis and aerobiosis (Figure 1). A minor band of Sod activity located halfway between M1 and M3 was observed and was consistent with formation of a hybrid Sod (Dougherty *et al.* 1978). Under anaerobiosis, a FeSod2 (M2) had about 7-fold increased relative activity when isolated from cells grown in the presence of $FeSO_4$ as compared with cells challenged with DTPA (compare lane 1 with lane 2). (For this experiment and the others described in this study, there was a variance of ± 20 to 25% in the densitometry data.) Cells grown aerobically in the presence of $FeSO_4$ had a 2-fold increase in the amount of FeSod2 as compared with cells grown in the presence of DTPA (compare lane 3 with lane 4). Very low levels of MnSod (M3) were produced in anaerobically grown cells and in $FeSO_4$-supplemented aerobic cultures (compare lanes 1, 2 and 3 with lane 4); about 4-fold higher relative intensity was observed from bacteria grown aerobically in the presence of DTPA than when cells were grown in the presence of $FeSO_4$ (compare lane 3 with lane 4). No significant increases in MnSod activity were found when cells were incubated aerobically with either 0, 25 or 100 μM MV (results not shown).

Crude extracts were prepared from cells harvested at selected phases of growth and analyzed by native PAGE and Sod-specific staining. When aerobically grown cells were diluted into fresh L broth contained in sealed culture flasks in the presence of $FeSO_4$ or DTPA, continued growth resulted in constant production of FeSod1 and decreased production of FeSod2 and MnSod (Figure 2A, compare lanes 1 with 4 and 5 with 9). By contrast, aerobically grown cells that were diluted in L broth with constant aeration produced a 2-fold increase in FeSod1 along with a 5-fold increase in FeSod2 in the presence of $FeSO_4$ and a 7-fold increase in FeSod1, a 2-fold increase in FeSod2 and constant amounts of MnSod in the presence of DTPA (Figure 2B,

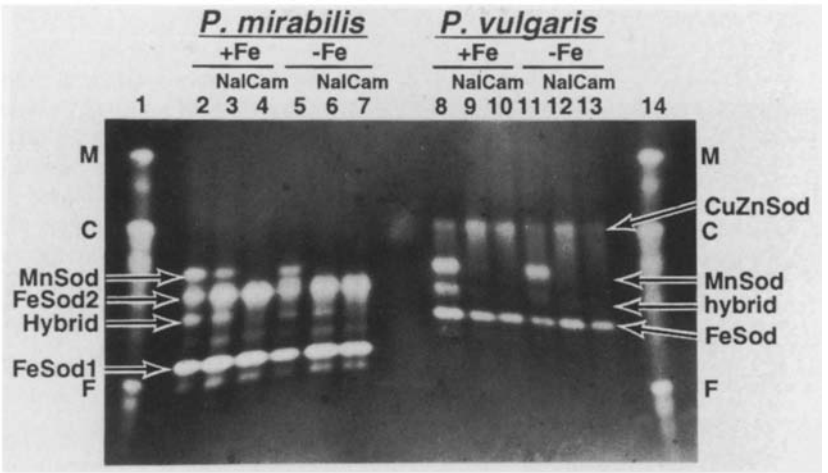

Figure 3. Inhibition of the aerobic induction of Sod production from *P. mirabilis* 7002 and *P. vulgaris* 33420. For *P. mirabilis* 7002 (lanes 2–7) and *P. vulgaris* 33420 (lanes 8–13), extracts in lanes 2–4 and 8–10 were from cells incubated in the presence of 100 μM $FeSO_4$, while extracts in lanes 5–7 and 11–13 were from cells incubated in the presence of 100 μM DTPA. Extracts in lanes 3, 6, 9 and 12 were from cells treated with 250 $\mu g\ ml^{-1}$ nalidixic acid (Nal). Extracts in lanes 4, 7, 10 and 13 were from cells incubated in the presence of 250 $\mu g\ ml^{-1}$ chloramphenicol (Cam). Lanes 1 and 14 contained 1 μg each of purified *E. coli* MnSod (M) and FeSod (F) and bovine CuZnSod (C). The data are representative of two independent trials.

compare lanes 1 with 4 and 5 with 9). When anaerobic cultures were diluted with fresh aerated L broth, nalidixic acid and chloramphenicol stopped cell growth and inhibited the DTPA-dependent decrease in FeSod1 (compare Figure 3, lanes 5 and 7) and the production of MnSod (compare Figure 3, lanes 2–4 and 5–7). Figure 4(A and B) summarizes the results of aerobically grown cultures diluted into fresh medium containing $FeSO_4$ or DTPA and various antibiotics with dioxygen insufficiency or sufficiency. Under air limitations (Figure 4A), chloramphenicol inhibited decreased production of MnSod (compare Figure 4A, lane 2 with 4, 10-fold; lane 5 with 7, 2-fold) and FeSod2 (compare Figure 4A, lane 5 with 7, 3-fold). The results illustrated in Figure 4(A, lanes 2 and 5) paralleled those presented in Figure 1 (lanes 1 and 2). By contrast with aerobic cultures and sufficient dioxygen (Figure 4B), chloramphenicol prevented a 75% decrease in FeSod1 (lanes 8 and 13), a 50% decrease in MnSod (lanes 2 and 7) and biosynthesis of FeSod2 (lanes 7 and 13). The latter observation was pursued; FeSod1 and MnSod were stable to prolonged incubation while production of FeSod2 in chloramphenicol-containing cultures decreased about 75% over a 2 h period (results not shown).

Sod production in P. vulgaris *33420*

Under anaerobic and aerobic growth conditions, FeSod (V1) and MnSod (V2) had similar behavior to that of FeSod1 (M1) and MnSod (M3) from *P. mirabilis* 7002 (Figure 1). For FeSod, there was 2-fold increased production from anaerobically grown cells with DPTA as compared with $FeSO_4$ (compare lane 5 with lane 6), 4-fold increased production from iron-supplemented aerobic cultures over that of anaerobic cultures (compare lane 5 with lane 7) and 50% decreased production when aerobic cultures were supplemented with DTPA (compare lane 7 with lane 8). Aerobic production of MnSod increased 4-fold when cells were supplemented with DTPA as compared with $FeSO_4$ (compare lane 7 with lane 8). A minor band of Sod activity located halfway between V1 and V2 was observed, and was consistent with formation of a hybrid Sod. A CuZnSod (V3) was produced in aerobically grown cells and had 4-fold increased relative activity when isolated from cells supplemented with $FeSO_4$ than from cells supplemented with DTPA (compare lane 7 with lane 8).

During growth under aerobic conditions, production of FeSod, MnSod, and CuZnSod increased through late exponential phase (Figure 2C, note there was increased smearing of Sod in extracts from late-exponential phase cultures). In the presence of DTPA, low levels of FeSod resulted during the initial incubation period with fresh medium (compare lane 7 with lane 11). When anaerobically grown cultures were diluted with fresh aerated L broth, nalidixic acid and chloramphenicol inhibited cell growth, iron- and DTPA-dependent changes in FeSod, and the production of MnSod (compare Figure 3, lanes 8–10 with 11–13). The short incubation time resulted in low levels of CuZnSod. Figure 4(C) summarizes the results of aerobically grown cultures diluted into fresh medium containing $FeSO_4$ or DTPA and various antibiotics with dioxygen sufficiency. Chloramphenicol prevented a decrease in FeSod (lanes 7 and 11), 50% decrease (lanes 2 and 6) and 2-fold increase in MnSod (lanes 7 and 11), and biosynthesis of active CuZnSod (lanes 6 and 11). The results illustrated in Figure 4(C, lanes 2 and 7) paralleled those presented in Figure 1 (lanes 7 and 8).

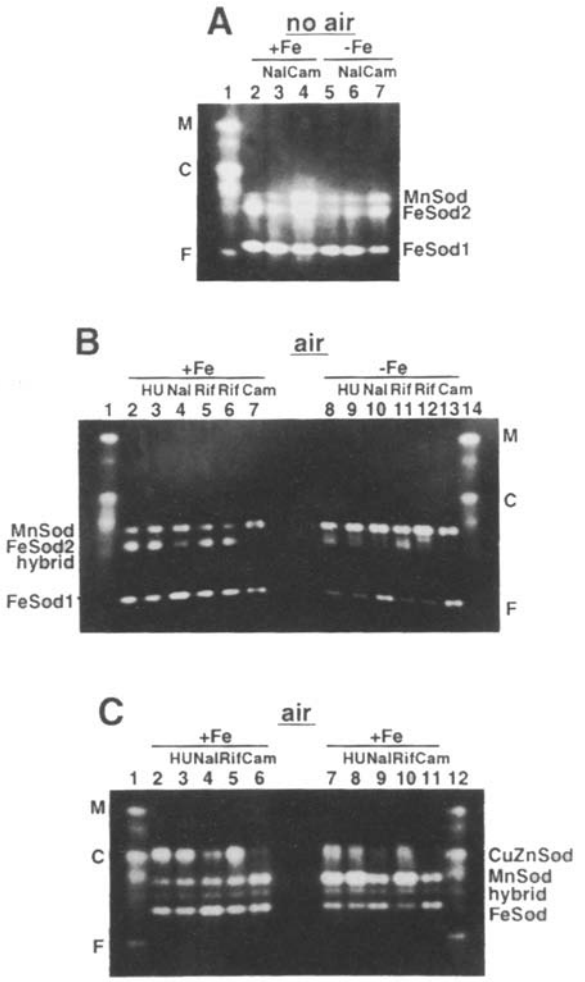

Figure 4. Transcription and translational inhibitors affect iron-dependent production of Sod activity from *P. mirabilis* 7002 (**A** and **B**) and *P. vulgaris* 33420 (**C**). For *P. mirabilis* 7002, extracts were prepared from cells incubated anaerobically (**A**) or aerobically (**B**) in the presence of 100 μM $FeSO_4$ (**A**, lanes 2–4; **B**, lanes 2–7) or 100 μM DTPA (**A**, lanes 5–7; **B**, lanes 8–13). Extracts in **A** lanes 3 and 6 and **B** lanes 4 and 10 were from cells treated with 250 $\mu g\,ml^{-1}$ nalidixic acid (Nal). Extracts in **B** lanes 3 and 9 were from cells treated with 250 $\mu g\,ml^{-1}$ hydroxyurea (HU). Extracts in **B** lanes 5 and 11 and lanes 6 and 12 were from cells treated with 50 or 250 $\mu g\,ml^{-1}$ rifampicin (Rif), respectively. Extracts in **A** lanes 4 and 7 and **B** lanes 7 and 13 were from cells incubated in the presence of 250 $\mu g\,ml^{-1}$ chloramphenicol (Cam). One microgram each of purified *E. coli* MnSod (M) and FeSod (F) and bovine CuZnSod (C) was loaded into lane 1 of **A** and lanes 1 and 14 of **B**. For *P. vulgaris* 33420, extracts were prepared from cell incubated aerobically (**C**) in the presence of 100 μM $FeSO_4$ (lanes 2–6) or 100 μM DTPA (lanes 7–11). Extracts in lanes 3 and 8 were from cells incubated in the presence of 250 $\mu g\,ml^{-1}$ HU. Extracts in lanes 4 and 9 were from cells treated with 250 $\mu g\,ml^{-1}$ nalidixic acid (Nal). Extracts in lanes 5 and 10 were from cells incubated in the presence of 250 $\mu g\,ml^{-1}$ rifampicin (Rif). Extracts in lanes 6 and 11 were from cells incubated in the presence of 250 $\mu g\,ml^{-1}$ chloramphenicol (Cam). Lanes 1 and 12 contained 1 μg each or purified *E. coli* MnSod (M) and FeSod (F) and bovine CuZnSod (C). The data are representative of two independent trials.

Discussion

The present studies yielded interesting results concerning the impact of metal ions and dioxygen on Sod production from *Proteus*. Iron played an important role in the increased production of FeSod and decreased production of MnSod, while aerobic growth contributed to MnSod production. While *Proteus* shared similarities with *E. coli* with respect to Sod production, there were several differences that may prove important in the survival of this class of urinary tract pathogens.

Iron-dependent growth in rich medium

Our results supported a role for iron in rapid growth of *Proteus*. When iron was restricted by the use of an effective chelator such as DTPA (Fe^{3+}, $\log K_{ML} = 28$), growth slowed. However, there was continued increase in cell density, which paralleled final turbidity values of iron-sufficient cultures. We noted that iron, manganese and amino acids allowed rapid growth of *Proteus* in defined minimal medium (Niederhoffer, unpublished results). Although we were unable to confirm catecholate- or hydroxamate-containing siderophore production in defined minimal medium, these observations were consistent with the production of low-affinity siderophores derived from the deamination of amino acids in rich medium (Evanylo *et al.* 1984, Drechsel *et al.* 1993).

Sod production in P. mirabilis *and* P. vulgaris

Few eubacterial systems have been examined for effects of dioxygen and metal ions on Sod expression; the best studied is that of *E. coli* (Niederhoffer *et al.* 1990, Hassan & Sun 1992, Wu & Weiss 1992, Compan & Touati 1993, Privalle & Fridovich 1993). Behavior of Sod in that system may be useful in dissecting the importance of Sod in *Proteus* as well as other pathogenic microorganisms. Although the *E. coli* FeSod and MnSod share high similarities at both the nucleotide and amino acid sequence level, the FeSod is expressed constitutively under both anaerobiosis and aerobiosis, while expression of MnSod is induced by aerobiosis and iron limitation. Direct interactions of holoFur, but not apoFur, with the *sodA* (MnSod) and *sodB* (FeSod) promoters along with ArcA binding to *sodA* has been demonstrated (Niederhoffer *et al.* 1990, Tardat & Touati 1993), which resulted in active exploration of regulatory events concerning *sodA* expression but relatively little information on *sodB*. Steinman has proposed distinct functions for FeSod and MnSod that would partially explain both the localization of Sod proteins and expression patterns (Hopkin *et al.* 1992, Steinman *et al.* 1994). Recent work from Fridovich and coworkers suggested that the CuZnSod may be easily overlooked because of its sensitivity to extract preparation methods (Benov & Fridovich 1994).

Proteus appeared to contain multiple Sod proteins (Figure 1), FeSod1, FeSod2 and MnSod for *P. mirabilis* 7002 and FeSod, MnSod and CuZnSod for *P. vulgaris* 33420. Specific

activities were typical for crude cellular extracts of other pathogenic bacteria (Hewitt & Morris 1975, Yano & Nishie 1978, Norrod & Morse 1979, Martin *et al.* 1986, Haas & Goebel 1992, Nakayama 1994). The FeSods were identified by inactivation in the presence of both H_2O_2 and KCN (Slykhouse & Fee 1976, Privalle & Gregory 1979), MnSod by its specific aerobic induction, while CuZnSod was identified by its sensitivity to KCN (Salin & McCord 1974). Figure 2 shows that Sod production from *P. mirabilis* and *P. vulgaris* depended on iron and growth phase, consistent with the presence of a regulated iron uptake system. Thus we observed that aerobic FeSod1 production in *P. mirabilis*, aerobic FeSod production in *P. vulgaris*, aerobic FeSod2 production in *P. mirabilis*, and aerobic MnSod and CuZnSod production in *P. vulgaris* increased from early through late exponential phase. Although the growth phase characteristics of the individual Sod proteins were consistent with the static studies illustrated in Figure 1, iron limitation coupled to the larger head space in the anaerobic culture flasks resulted in incomplete repression of *P. mirabilis* 7002 FeSod2 and MnSod. The present results, which were derived from protein production, contrast those of *E. coli sodB'-'lacZ* and *sodA'-'lacZ* (Niederhoffer *et al.* 1990), where the FeSod gene fusion is dependent on the level of soluble iron but independent of growth phase and the MnSod gene fusion is dependent on both soluble iron concentration and growth phase.

Our results were based upon measuring Sod activities from various growing cultures. In order to assess whether there were qualitative effects at the level of gene expression, cultures were incubated in the presence of iron or DTPA and specific antibiotic inhibitors of DNA replication, DNA transcription and protein synthesis. Only nalidixic acid and chloramphenicol inhibited cell growth. Nalidixic acid, which influences *E. coli sodA* transcriptional activity through changes in DNA topology (Schrum & Hassan 1992), and chloramphenicol, which inactivates peptidyl transferase, inhibited the DTPA-dependent decreased aerobic production of FeSod and the iron-dependent decreased anaerobic and aerobic production of MnSod (Figure 4) and the DTPA-dependent aerobic production of FeSod and the iron-dependent aerobic production of MnSod (Figure 3). Interestingly, chloramphenicol treatment resulted in very low levels of FeSod2 from *P. mirabilis* 7002 and CuZnSod from *P. vulgaris* 33420 supplemented with $FeSO_4$ or DTPA, a result that indicated lessened protein stability during dilution and incubation in fresh medium. Anaerobic cultures of *P. mirabilis* and *P. vulgaris* that were exposed to air and supplemented with $FeSO_4$ or DTPA expressed little MnSod when chloramphenicol (protein synthesis inhibitor) was added (Figure 3). Parallel studies on cultures treated with nalidixic acid produced low levels of MnSod especially with DTPA in the growth medium. Production of FeSod and CuZnSod were relatively unaffected, although we observed the predicted iron-dependent repression of FeSod from *P. vulgaris*. Because of the short incubation time, CuZnSod was not optimally expressed in aerobic cultures. Control studies were conducted to assess the effect of incubation time on stability of crude cell Sod activities. Over a 2 h period, no significant changes were observed in specific Sod activities as visualized on polyacrylamide gels (results not shown). In addition, individual Sod activities were stable to prolonged incubation with DTPA, which supported kinetically stable metal ion centers *in vitro*. Although we do not have direct evidence for transcriptional control of Sod production, these results were consistent with a current model of iron and redox regulation of *sod* genes in *E. coli* (Compan & Touati 1993). In addition, our results shared similarities with those involving *E. coli* Sod previously observed by Hassan and coworkers (Moody & Hassan 1984).

A productive approach to controlling infection and eliminating disease would be to understand host defense factors and pathogen physiology. Under typical conditions found in the urinary tract, low iron and microaerobiosis, there would be FeSod1, FeSod2, MnSod and CuZnSod production. However, within a mature biofilm one would anticipate sufficient iron levels and dioxygen limitation, resulting in FeSod1, FeSod2 and FeSod production. How iron, dioxygen and Sod fit toegher in pathogen physiology remains to be determined through molecular characterization of regulatory loci and *sod* expression during cellular invasion.

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