JOURNAL OF BACTERIOLOGY, Oct. 2005, p. 6678–6682 Vol. 187, No. 19
0021-9193/05/$08.00+0 doi:10.1128/JB.187.19.6678–6682.2005

DOS_{Ec}, a Heme-Regulated Phosphodiesterase, Plays an Important Role in the Regulation of the Cyclic AMP Level in *Escherichia coli*‡

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Heme-regulated phosphodiesterase from *Escherichia coli* (DOS_{Ec}) catalyzes the hydrolysis of cyclic AMP (cAMP) in vitro and is regulated by the redox state of the bound heme. Changes in the redox state result in alterations in the three-dimensional structure of the enzyme, which is then transmitted to the functional domain to switch catalysis on or off. Because DOS_{Ec} was originally cloned from *E. coli* genomic DNA, it has not been known whether it is actually expressed in wild-type *E. coli*. In addition, the turnover number of DOS_{Ec} using cAMP as a substrate is only 0.15 min^{-1}, which is relatively low for a physiologically relevant enzyme. In the present study, we demonstrated for the first time that the DOS_{Ec} gene and protein are expressed in wild-type *E. coli*, especially under aerobic conditions. We also developed a DOS_{Ec} gene knockout strain (Δ*dos*). Interestingly, the knockout of *dos* caused excess accumulation of intracellular cAMP (26-fold higher than in the wild-type strain) under aerobic conditions, whereas accumulation of cAMP was not observed under anaerobic conditions. We also found differences in cell morphology and growth rate between the mutant cells and the wild-type strain. The changes in the knockout strain were partially complemented by introducing an expression plasmid for *dos*. Thus, the present study revealed that expression of DOS_{Ec} is regulated according to environmental O_2 availability at the transcriptional level and that the concentration of cAMP in cells is regulated by DOS_{Ec} expression.

As described by Delgado-Nixon et al. (6), the heme-regulated phosphodiesterase from *Escherichia coli* (DOS_{Ec}) is composed of an N-terminal heme-bound PAS domain and a C-terminal phosphodiesterase domain. The phosphodiesterase activity of this enzyme hydrolyzes adenosine 3′,5′-cyclic monophosphate (cAMP) when the heme iron is in the ferrous (Fe^{2+}) but not in the ferric (Fe^{3+}) state (18). Changes in the redox state of the heme-bound iron are transduced to the enzyme's catalytic domain, thus regulating the catalytic activity (6, 12, 18, 20, 21, 24). Crystallographic analysis has revealed that reduction of the heme iron induces a global conformational change in the FG loop within the heme-binding domain and causes the replacement of a heme-bound water with a side chain of Met-95 (12). These profound structural changes in the PAS domain accompanied by the heme redox change are transmitted to the phosphodiesterase domain so that the heme redox state can act as an on/off switch for the enzyme. Thus, DOS_{Ec} can be classified as a heme-based sensor.

DOS_{Ec} was originally identified in the *E. coli* genomic DNA sequence (6). Despite detailed biochemical and biophysical studies (6, 12, 18, 20, 21, 24), it has been unclear whether the DOS_{Ec} protein and gene (*dos*) are actually expressed in wild-type *E. coli*. In addition, the turnover number of DOS_{Ec} using cAMP as a substrate is only 0.15 min^{-1}, which is relatively low for a physiological enzyme and brings into question whether it plays a role in cAMP regulation in vivo. Therefore, further studies are needed to examine the expression of the DOS_{Ec} protein and gene in wild-type *E. coli* cells and to determine its physiological role.

In the present study, we examined the transcriptional level of *dos* and expression of the DOS_{Ec} protein in wild-type *E. coli* under both aerobic and anaerobic conditions. Because knockout strains can be very useful for elucidating the physiological roles of proteins, we constructed a *dos* knockout strain and examined its phenotype, including the relative intracellular concentration of cAMP.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized at the Nihon Gene Research Laboratory (Sendai, Japan) and Kurabo Industries (Osaka, Japan). Restriction and modifying enzymes for DNA recombination were purchased from Takara Bio (Otsu, Japan), Toyobo (Osaka, Japan), New England Biolabs (Beverly, MA), and Roche Diagnostics (Basel, Switzerland). Other chemicals were of the highest available quality from Wako Pure Chemicals (Osaka, Japan) and were used without further purification.

Bacterial growth. For aerobic growth, the overnight culture was diluted 1:1,000 in 50 ml LB medium and grown in a 300-ml culture bottle within an air incubator at 37°C with vigorous shaking. For anaerobic growth, cells were diluted 1:1,000 in 15 ml LB medium in a 20-cm test tube that was placed in a water bath at 37°C and was continuously bubbled with N_2. Growth was followed by measuring the optical density at 600 nm (OD_{600}). Cells reaching the stationary phase (OD_{600} of approximately 5.0 and 1.2 for aerobic and anaerobic growth, respectively) were used for further analyses.

Real-time reverse transcription (RT)-PCR for quantification of DOS_{Ec} mRNA. Isolation of total RNA was performed with the RNAgents Total RNA isolation system (Promega, Madison, WI) according to the manufacturer's pro-

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‡ Supplemental material for this article may be found at http://jp.asm.org/.

TABLE 1. Primers used for analysis of transcription by real-time RT-PCR and construction of Δ*dos* strains

Gene	Primer	Sequence[a]	Amplified PCR product (bp)
dos	DOS-real-time1	5′-AAAACTCAAACCGGATCAG-3′	141
	DOS-real-time2	5′-AGCCGATAATGATTGACGA-3′	
Cm^r	Cm^r-F	5′-CTGGATCCCCGCGGCGCAGAAAAAAAGGATCT-3′	1,056
	Cm^r-R	5′-TAGGATCCCGGGGAGAGGCGGTTTGC-3′	

[a] Underlined sequences indicate restriction sites.

tocol. To minimize degradation of RNA, we collected grown cells after rapidly chilling them in ice-water, and all steps were performed on ice as quickly as possible. Total RNA was treated with RNase-free DNase (Takara Bio) with recombinant RNasin RNase inhibitor (Promega) for 30 min at 37°C to remove genomic DNA. Removal of enzymes and purification of total RNA were performed with an RNeasy MinElute cleanup kit (QIAGEN, Hilden, Germany). The quantity of purified total RNA was estimated using the absorbance at 260 nm measured with a Shimadzu UV-2200 spectrophotometer.

Equal amounts (1 μg per reaction) of total RNA were reverse-transcribed using a First-Strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ). Design and synthesis of primers for real-time PCR, optimization of PCR conditions with the LightCycler (Roche Diagnostics), and real-time PCR in the presence of the DNA-binding fluorescent dye SYBR green were performed at the Nihon Gene Research Laboratory. The primer sequences are listed in Table 1. The results of real-time PCR were converted into copy numbers by comparison with a standard curve that was derived by simultaneously performing PCR assays with known concentrations of the target gene.

Immunoblot analysis. To generate an antibody against DOS_{Ec}, we expressed and purified full-length wild-type DOS_{Ec} protein as described previously (18). A rabbit polyclonal anti-DOS_{Ec} antibody was produced by Nikka Techno Service (Hitachi, Japan). Antiserum was bound to rProtein A Sepharose Fast Flow (Amersham Biosciences) equilibrated with a solution of 1.5 M glycerol and 3 M NaCl. The antibodies were then eluted with a solution of 0.1 M citrate and 0.5 M NaCl and then dialyzed against phosphate-buffered saline (20 mM sodium phosphate, pH 7.4, and 150 mM NaCl).

JM109 cells were harvested under aerobic and anaerobic conditions as described above. Equal amounts of both cell types were collected based on their OD_{600} and then were washed and suspended in phosphate-buffered saline. The cells were sonicated, and the soluble and insoluble fractions were separated by centrifugation at 100,000 × *g*. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrophoretically transferred to an Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). After blocking with 5% skim milk in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 1% Tween 20), the membrane was incubated for 1 h with anti-DOS_{Ec} antibody diluted in TBS-T and then for 1 h with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Cell Signaling Technology, Beverly, MA). The immunoreactive proteins were detected with ECL Western blotting detection reagents (Amersham Biosciences).

Construction of an *E. coli dos* knockout strain. The chloramphenicol resistance gene was amplified from pHSG396 (Takara Bio) with primers Cm^r-F and Cm^r-R, which include BamHI and SacII sites (Table 1). The PCR product was digested with BamHI and ligated into pKF19 (Takara Bio). The SacII-BamHI fragment of pET28a wild-type full-length *dos* (codons 70 to 340) was replaced by the SacII- and BamHI-digested Cm^r fragment from pKF19-Cm^r. The interrupted gene (Δ*dos*::Cm^r) was amplified by PCR with the primers for full-length *dos* (18, 24) and electroporated into *E. coli* JC7623 (*recBC sbcB*), a strain that shows high-frequency double-crossover homologous recombination (2, 11) and lacks exonuclease V, which digests linear DNA (3, 5). Next, growth was selected on LB containing 35 μg/ml of chloramphenicol. The Δ*dos*::Cm^r allele was introduced into *E. coli* W3110 and BL21(DE3) by phage transduction using $P1_{vir}$ phage (11, 13, 14). Gene disruption was confirmed by PCR.

Complementation of Δ*dos* BL21(DE3) cells with the pET28a-DOS_{Ec} expression vector. *E. coli* Δ*dos* BL21(DE3) was transformed with the expression vector pET28a-WT DOS_{Ec} (18, 24). Bacteria containing the pET28a-DOS_{Ec} expression vector were selected on LB-agar containing 35 μg/ml of chloramphenicol and 50 μg/ml of kanamycin because the Δ*dos* strain is resistant to chloramphenicol and pET28a encodes kanamycin resistance. LB medium was inoculated at 37°C with Δ*dos* BL21(DE3) containing the pET28a-DOS_{Ec} expression vector, Δ*dos* BL21(DE3), or wild-type BL21(DE3). When the OD_{600} reached 0.6, the cultures were adjusted to 0.05 mM isopropyl-β-D-thiogalactopyranoside and 0.45 mM δ-aminolevulinic acid. The cells were then incubated overnight at 37°C under aerobic conditions.

Normalization of cell number. We first examined the relationship between the OD_{600} and the number of colonies for both wild-type W3110 and Δ*dos* W3110 strains so that the OD_{600} could be used to obtain equal cell numbers. The same numbers of wild-type W3110 and Δ*dos* W3110 strains from overnight cultures were used for monitoring growth rates under aerobic and anaerobic conditions. Cells were grown under aerobic and anaerobic conditions as described above. Cells reaching the stationary phase were used for microscopic observation and cAMP quantification.

Determination of the Intracellular cAMP concentration. Extraction and determination of the intracellular cAMP concentration were performed using a cAMP Biotrak Enzyme Immunoassay System (Amersham Biosciences). The assay is based on competition between unlabeled cAMP and a fixed quantity of peroxidase-labeled cAMP for a limited number of binding sites on a cAMP-specific antibody. Addition of peroxidase substrate (3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide) generates a colored product that absorbs at 630 nm. A series of dilutions containing 2 to 128 fmol cAMP were prepared as standards. According to the manufacturer's protocol, we calculated the percentage bound ($\%B/B_0$) for each standard and sample as follows: $\%B/B_0$ = 100 × (OD of standard or sample –OD for nonspecific binding in the absence of anti-cAMP) ÷ (OD for no cAMP –OD for nonspecific binding in the absence of anti-cAMP). A standard curve was generated by plotting $\%B/B_0$ as a function of the fmol of cAMP standard.

RESULTS

DOS_{Ec} gene is expressed in wild-type *E. coli* cells. The transcriptional level of the DOS_{Ec} gene in aerobically and anaerobically grown wild-type *E. coli* W3110 was examined by real-time RT-PCR. Copy numbers were estimated to be 4,020 and 1,530 per 30 ng of total RNA from *E. coli* grown under aerobic and anaerobic conditions, respectively. Thus, we confirmed that the DOS_{Ec} gene is transcribed in the wild-type strain, and we found that the copy number is approximately 2.6-fold higher in aerobically grown cells than in anaerobically grown cells.

DOS_{Ec} protein is expressed predominantly in aerobically grown wild-type *E. coli* cells. We next examined the level of DOS_{Ec} protein expression in the wild-type *E. coli* strain by immunoblotting with an anti-DOS_{Ec} polyclonal antibody. As

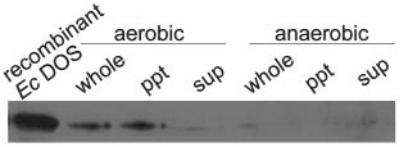

FIG. 1. Expression of the DOS_{Ec} proteins in *E. coli* cells. Immunoblot of the DOS_{Ec} protein in soluble and insoluble fractions of aerobically and anaerobically grown cells. Purified recombinant DOS_{Ec} was used as a positive control. Whole, whole-cell extract; ppt, precipitates obtained by sonication followed by centrifugation at 100,000 × *g*; sup, supernatant fraction obtained by sonication followed by centrifugation at 100,000 × *g*.

TABLE 2. Concentration of intracellular cAMP in aerobically and anaerobically grown wild-type W3110, Δ*dos* W3110, BL21(DE3)/pET28a-WT DOS_{Ec}, and wild-type BL21(DE3) cells

Growth conditions	Intracellular cAMP concn (fmol/10^8 cells)					
	Δ*dos* W3110	Wild-type W3110	Δ*dos*/wild-type ratio	BL21(DE3)/pET28a-WT DOS_{Ec}	Wild-type BL21(DE3)	Wild-type/ pET28a-DOS_{Ec} ratio
Aerobic	314.4	11.9	26	3.4	17.5	5.2
Anaerobic	1.4	0.8	1.8			

shown in Fig. 1, an immunoreactive protein band with a position similar to that of the purified recombinant DOS_{Ec} was observed in whole extracts of aerobically grown cells. In contrast, the concentration of DOS_{Ec} was very low in anaerobically grown cells. For aerobically grown cells, most of the band ascribed to DOS_{Ec} appeared to be present in the insoluble fraction, and very little was present in the soluble fraction.

Level of intracellular cAMP is elevated in aerobically but not anaerobically grown Δ*dos* cells. DOS_{Ec} hydrolyzes cAMP to 5′-AMP in vitro with a turnover number (0.15 min^{-1}) (18, 24) that is low compared with other physiologically important enzymes. If cAMP is a physiological substrate, the intracellular cAMP concentration would be expected to be higher in *dos*-null cells. To examine this possibility, we generated cells in which the *dos* gene was knocked out (Δ*dos*) and measured the intracellular cAMP concentration of wild-type and Δ*dos* W3110 cells by immunoassay (Table 2). Interestingly, we found that the intracellular cAMP concentration in the aerobically grown Δ*dos* cells is about 26-fold higher than in the wild-type cells. On the other hand, the intracellular cAMP concentrations in the anaerobically grown Δ*dos* and wild-type cells were similar. These results suggested that the cAMP level is regulated by DOS_{Ec} in wild-type *E. coli*.

The concentration of cAMP is lower in the DOS_{Ec}-overexpressing strain than the wild-type strain. We next examined the level of intracellular cAMP in aerobically grown BL21(DE3) overexpressing DOS_{Ec} and wild-type BL21(DE3). As seen in Table 2, the cAMP level of BL21(DE3) overexpressing DOS_{Ec} was 5.2-fold lower than in the wild-type BL21(DE3). Two separate wild-type strains had similar cAMP levels. These results are consistent with the findings from the Δ*dos* strain, suggesting again that the cAMP level is regulated by DOS_{Ec} in vivo.

Cell morphology and growth rate of Δ*dos* and wild-type cells differ only under aerobic conditions. We found that, when grown under aerobic conditions, the Δ*dos* cells were longer than the wild-type strains, indicating that cell filamentation occurred in the *dos* knockout strains (Fig. 2). Cell filamentation was observed in two strains: aerobically grown Δ*dos* W3110 (Fig. 2B) and Δ*dos* BL21(DE3) (Fig. 2D). In contrast, like wild-type cells (Fig. 2E), anaerobically grown Δ*dos* W3110 did not show filamentation (Fig. 2F). On the other hand, overexpression of DOS_{Ec} under aerobic conditions caused minicell formation (Fig. 2G). Complementation of the deleted DOS_{Ec} gene by transformation with the pET28a-WT DOS_{Ec} expression resulted in partial rescue of phenotype of the wild-type BL21(DE3) strain (Fig. 1S).

The growth characteristics of Δ*dos* W3110 and wild-type W3110 were monitored (Fig. 3). Under aerobic conditions (Fig. 3A), the rate of growth of the Δ*dos* W3110 cells was lower than that of the wild-type W3110 cells during both the initial and logarithmic phases. In addition, the number of cells in the stationary phase was lower for Δ*dos* W3110 than for the wild-type W3110. In contrast, the growth rates of both Δ*dos* and wild-type W3110 cells were similar under anaerobic conditions (Fig. 3B).

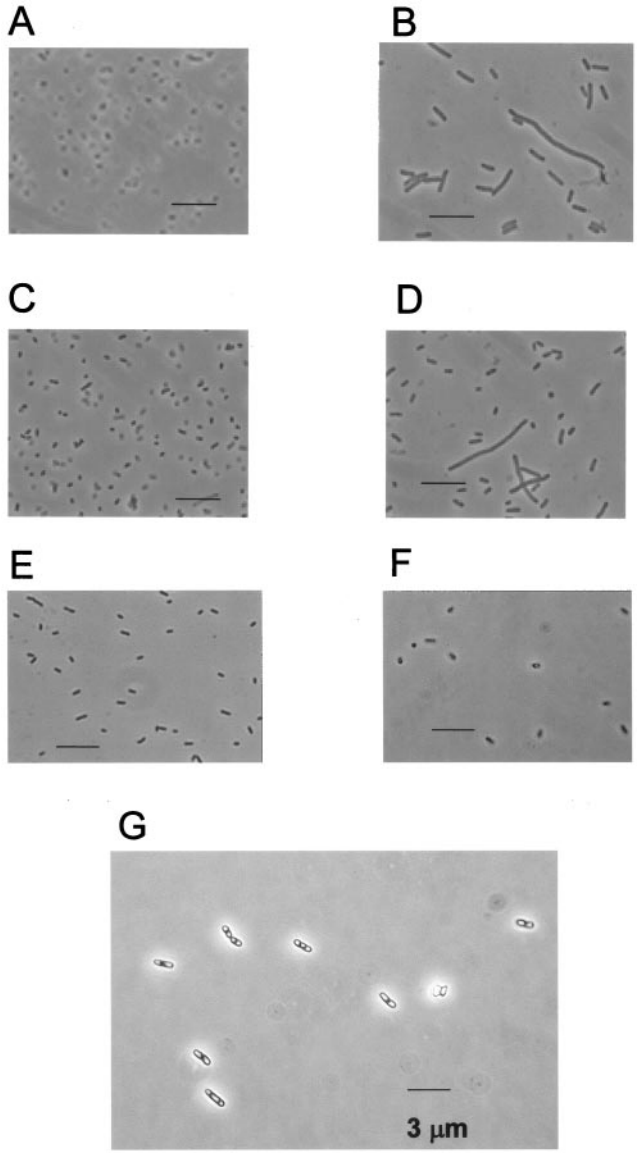

FIG. 2. Morphology of aerobically and anaerobically grown wild-type, Δ*dos*, and DOS_{Ec}-overexpressing strains. Shown are phase contrast images of wild-type W3110 (A and E), Δ*dos* W3110 (B and F), wild-type BL21(DE3) (C), Δ*dos* BL21(DE3) (D), and BL21(DE3) transformed with pET28a-WT-DOS_{Ec} (G). In A to D and G, cells were grown under aerobic conditions, and in E and F, cells were grown under anaerobic conditions. Bar, 10 μm in A to F and 3 μm in G.

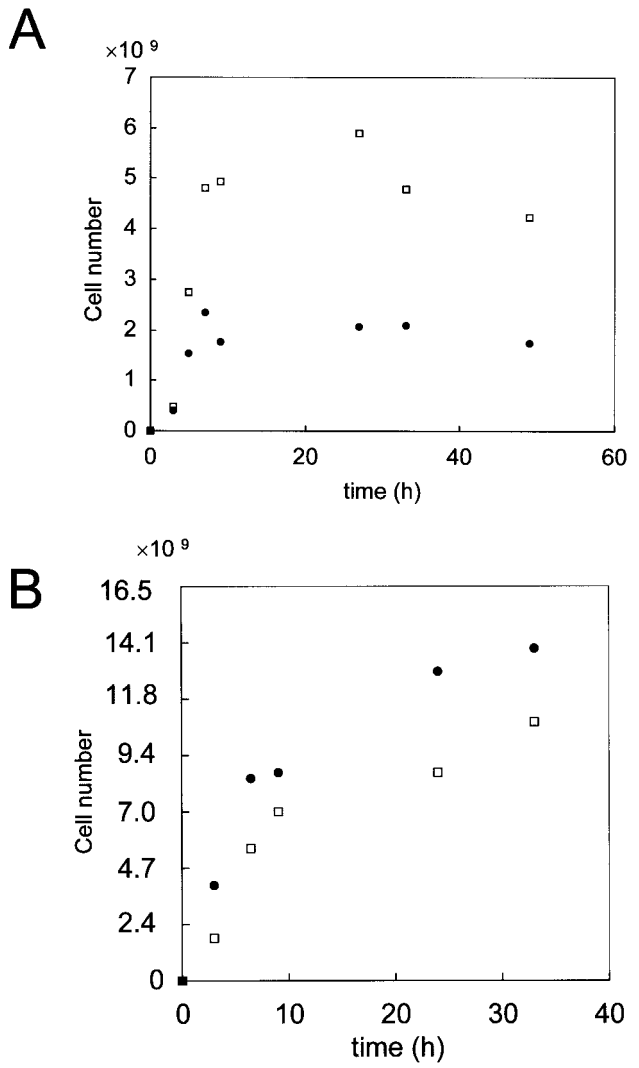

FIG. 3. Growth rates of Δ*dos* W3110 and wild-type W3110 under (A) aerobic and (B) anaerobic conditions. Solid circle, Δ*dos* W3110; open square, wild-type W3110.

DISCUSSION

The aim of the present study was to characterize the expression and the physiological role of the heme-regulated phosphodiesterase DOS_{Ec}. To help determine its function, we examined the effect of a gene knockout for DOS_{Ec}.

Knockout of DOS_{Ec} increases the level of cAMP in *E. coli*. In the present study, we showed that, when grown under aerobic conditions, Δ*dos* W3110 cells contain a 26-fold higher intracellular concentration of cAMP than wild-type W3110 cells. A difference in cAMP levels was not observed under anaerobic conditions. Furthermore, knockout of the DOS_{Ec} gene induced filamentation. On the other hand, mini-cell formation and reduction of the cAMP level were observed in cells overexpressing DOS_{Ec}. Interestingly, constitutive expression of *cya*, a gene encoding adenylate cyclase (cAMP synthase), has been reported to cause filamentation in *E. coli* cells due to elevated intracellular cAMP (23). These filaments are divided into rods as the intracellular cAMP level is decreased (22). Also, recent studies using a protein microarray showed that cAMP binds DOS_{Ec} in vitro (19). These results indicate a correlation between the morphology and the intracellular cAMP concentration in *E. coli*, and they indicate that cAMP is a potential substrate for DOS_{Ec} in vivo.

However, in vitro, the activity of DOS_{Ec} is relatively low (0.15 min^{-1}) compared with *Acetobacter xylinum* PDEA1 (90 min^{-1}), a homologous protein whose substrate is cyclic diguanylic acid (4, 6, 8). This implies that there are other factors that enhance the activity of DOS_{Ec} in vivo. In fact, the O_2 sensor protein *Rhizobium meliloti* FixL, whose heme-PAS domain is homologous with that of DOS_{Ec}, interacts with FixT, which regulates its kinase activity independently of O_2 binding to heme (7). In addition, it is possible that DOS_{Ec} catalyzes cyclic diguanylic acid degradation in vivo and that regulation of cAMP level is a secondary effect. We are now attempting to identify the partner protein(s) of DOS_{Ec}, and we are investigating the possibility that cyclic diguanylic acid is an alternative substrate for this enzyme.

It might be expected that the knockout of DOS_{Ec} would cause an even larger increase in intracellular cAMP than we observed, but *E. coli* has another major cAMP phosphodiesterase, CpdA (1, 10, 15–17). This second phosphodiesterase requires free Fe^{2+} for catalytic activity (10, 17), whereas DOS_{Ec} requires Mg^{2+} (18). Because they do not compete with each other, CpdA may function in the Δ*dos* strain to reduce the cAMP level. If both genes were deleted, intracellular cAMP concentrations might be much higher than we observed in the Δ*dos* strains.

Environmental O_2 concentration affects the expression of the DOS_{Ec} protein but does not affect its redox state. *E. coli* is a facultative anaerobic bacterium that uses different metabolic pathways according to environmental O_2 availability. The present study showed marked protein expression of DOS_{Ec} under aerobic conditions but little protein expression under anaerobic conditions. The cell morphology, cell growth rate, and intracellular cAMP concentration of the Δ*dos* and wild-type strains reflected this redox-dependent expression. Therefore, it is obvious that transcription of the DOS_{Ec} gene is regulated by environmental O_2 availability. Many redox sensor proteins, including transcriptional factors, have been proposed in *E. coli* (9), but a database on transcriptional regulation and operon organization in *E. coli* K-12 (http://www.cifn.unam.mx/ComputationalGenomics/regulondb/) suggests that none of them regulate the transcription of *dos*. Therefore, an unknown redox-dependent factor may regulate the transcription of *dos* in *E. coli*.

The phosphodiesterase activity of DOS_{Ec} is only active when the heme iron bound to PAS-A is in the reduced (Fe^{2+}) state (18, 24). Unexpectedly, however, cAMP was hydrolyzed even in cells grown under aerobic conditions. Therefore, it is possible that the changes in cAMP reported here are due to an indirect effect of DOS_{Ec}. Also, it has been reported that DOS_{Ec} may be in the reduced form in vivo (8). Furthermore, we speculate that DOS_{Ec} is constitutively active as a result of reduction by unknown reductases or chemicals independent of the environmental O_2 availability.

ACKNOWLEDGMENTS

E. coli JC7623 and W3110 and the $P1_{vir}$ phage for construction of the Δ*dos* strain were kindly provided by Akiko Nishimura at the National

Institute of Genetics. We are grateful to Kiwamu Minamisawa at Tohoku University and Tetsuhiko Yoshimura at the Institute for Life Support Technology, Yamagata Public Corporation for the Development of Industry, for use of their phase contrast microscopes.

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