Dual regulation of catecholate siderophore biosynthesis in Azotobacter vinelandii by iron and oxidative stress

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Azotobacter vinelandii forms both catecholate and azotobactin siderophores during iron-limited growth. Azotobactin is repressed by about 3 µM iron, but catecholate siderophore synthesis continues up to a maximum of 10 µM iron. This suggests that catecholate siderophore synthesis is regulated by other factors in addition to the ferric uptake repressor (Fur). In this study the first gene required for catecholate siderophore biosynthesis, which encodes an isochorismate synthase (csbC), was isolated. The region upstream of csbC contained a typical σ70 promoter, with an iron-box overlapping the −35 sequence and a Sox-box (Box 1) overlapping the −10 sequence. Another Sox-box was found further upstream of the −35 sequence (Box 2). Also upstream, an unidentified gene (orfA) was detected which would be transcribed from a divergent promoter, also controlled by an iron-box. The activity of csbC and a csbC::luxAB fusion was negatively regulated by iron availability and upregulated by increased aeration and by superoxide stress. The iron-box in the csbC promoter was 74% identical to the Fur-binding consensus sequence and bound the Fur protein of Escherichia coli with relatively high affinity. Both Box 1 and Box 2 were in good agreement with the consensus sequence for binding the SoxS protein of E. coli and Box 1 was in very good agreement with the Sox-box found in the fpr promoter of A. vinelandii, which is also regulated by superoxide stress. Both Sox-boxes bound a protein found in A. vinelandii cell extracts, with Box 1 exhibiting the higher binding affinity. The Sox protein identified in this assay appeared to be constitutive, rather than inducible by superoxide stress. This indicates that the Sox response in A. vinelandii is different from that in E. coli. These data support the hypothesis that catecholate siderophore biosynthesis is under dual control, repressed by a Fur–iron complex and activated by another DNA-binding protein in response to superoxide stress. The interaction between these regulators is likely to account for the delay in ferric repression of catecholate siderophore production, since these siderophores have an additional role to play in the protection of iron-limited cells against oxidative damage.

Keywords: Azotobacter vinelandii, catecholate siderophores, isochorismate synthase, Fur, superoxide stress

INTRODUCTION

Azotobacter vinelandii is a Gram-negative, obligate aerobe capable of free-living nitrogen fixation. The oxygen-labile nitrogenase remains functional because of ‘respiratory protection’, where O₂ consumption is uncoupled from ATP generation and the dissolved O₂ concentration around the cell is reduced to very low levels (Robson & Postgate, 1980). Iron is an essential nutrient for A. vinelandii and is required for respiratory protection, for nitrogenase activity and for protection against toxic oxygen products generated by active respiration. In its native soil environment, A. vinelandii is quite capable of extracting iron from the insoluble...
iron minerals that abound under aerobic, neutral-pH conditions (Page & Huyer, 1984). The bacterium releases four siderophores for the solubilization, chelation and transport of iron into the cell. These include the catecholates azotochelin (Corbin & Bulen, 1969), aminochelin (Page & von Tigerstrom, 1988) and protochelin (Cornish & Page, 1995), as well as the pyoverdin-like siderophore azotobactin (Demange et al., 1986). These siderophores are produced under iron-limited conditions, but are expressed in a unique sequential fashion (Page & Huyer, 1984). At limiting iron levels less than 10 µM, the catecholates are produced, followed by azotobactin at less than 3 µM iron (Page & von Tigerstrom, 1988).

Respiration by iron-limited cells presents a considerable hazard: not only are superoxide radicals and hydrogen peroxide generated, but also iron-limited cells have very low superoxide dismutase (SOD) activity (Cornish & Page, 1998). Vigorous aeration of iron-limited A. vinelandii cultures causes the upregulation of catecholate siderophore synthesis, while azotobactin is not similarly affected (Cornish & Page, 1998). It has been shown that the generation of hydroxyl radicals by the iron-catalysed Fenton reaction is effectively limited when iron is chelated to azotochelin or protochelin (Cornish & Page, 1998). Therefore, it has been hypothesized that catecholate siderophores could play an additional role in preventing oxidative damage to iron-limited cells and that catecholate siderophore synthesis may be under the dual regulation of iron and oxidative stress.

The expression of high-affinity, siderophore-mediated uptake systems for iron accumulation is controlled at the level of transcription by the dissociation of the ferric uptake regulator (Fur) from an iron-box operator sequence (de Lorenzo et al., 1987). In iron-limited medium, decreased levels of the corepressor, intracellular Fe(III), allow Fur dimer dissociation from the promoter region and ensuing transcription of iron-regulated genes (Bagg & Neilands, 1987). This is a highly conserved control mechanism, found in most aerobes and facultative aerobes (Achenbach & Yang, 1997). Thus differential control of A. vinelandii siderophore synthesis could be explained by differences in Fur affinity for catecholate or azotobactin Fur-binding operator sequences. Unfortunately, siderophore-specific operator sequences have not been identified in A. vinelandii and there has been no proof of the existence of a Fur homologue in this organism.

Escherichia coli deals with oxidative stress through the increased transcription of genes activated by SoxS in response to superoxide or OxyR in response to hydrogen peroxide (Demple, 1996). Since SOD activity is low in iron-limited A. vinelandii, it is most likely that a superoxide stress (Sox) response may be involved. Such a control system is plausible, since recent work presents strong evidence for a superoxide-dependent regulator in A. vinelandii (Isas et al., 1995; Yannone & Burgess, 1997, 1998).

In this study we describe the regulation of the first gene in the A. vinelandii catecholate siderophore biosynthesis operon, which encodes an isochorismate synthase, and report its dual regulation by iron and oxidative stress.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The Azotobacter vinelandii strains studied were: wild-type UW (ATCC 13705), and the isogenic strains F196 (Tn5luxAB mutant, catecholate-minus; Sevinc & Page, 1992) and LM100 (ferredoxin I-minus: Martin et al., 1989). The Escherichia coli strains were VCS257 (cosmid cloning host, Stratagene), BN4020 (Fur+) and BN402 (Fur-) (Bagg & Neilands, 1987), HB101 and DH5α (Sambrook et al., 1989). Cloning vectors and plasmid constructs are listed in Table 1. A. vinelandii strains were maintained in Burk’s medium containing 1% (w/v) glucose and 0.11% ammonium acetate (Page & Sadoff, 1976). The iron content of the medium was varied by the addition of ferric citrate and cultures were grown in acid-washed glassware (Page, 1993). Liquid cultures were aerated on a platform shaker (New Brunswick Scientific model G-76) at 225 r.p.m. at 28–30 °C. E. coli strains were grown in Luria–Bertani (LB) medium (Sambrook et al., 1989) at 37 °C. The medium was made iron-sufficient by the addition of 300 µM ferric citrate. Antibiotics were added to the medium as required: ampicillin, 80 µg ml⁻¹; chloramphenicol, 50 µg ml⁻¹; kanamycin, 50 µg ml⁻¹ (E. coli) or 12.5 µg ml⁻¹ (A. vinelandii); tetracycline, 50 µg ml⁻¹.

**Siderophore analysis.** Cell growth was measured as OD₆₀₀ or as total cellular protein (Page & Huyer, 1984). Catecholate and azotobactin siderophores were detected by their absorbance at 310 nm or 380 nm, respectively, in acidified culture supernatants fluids (Page & Huyer, 1984), using a Hitachi U-2000 recording spectrophotometer. Catecholate siderophores were also quantitated by the Barnum assay (Barnum, 1977).

**Cell-free extract preparation and SOD assay.** A. vinelandii strain UW was harvested and lysed in a French pressure cell to prepare a cell-free extract (CFX) (Cornish & Page, 1998). One unit of SOD activity was defined as the amount of protein required to inhibit the maximal rate of nitro blue tetrazolium reduction by 50% (Cornish & Page, 1998; Oberley & Spitz, 1985). Values reported are means calculated from at least duplicate assays in which the standard deviation was not greater than 10%.

**Bioluminescence (Lux) activity in liquid cultures.** A. vinelandii strain F196 was grown for 16 h in Burk’s medium containing varied amounts of ferric citrate. Methyl viologen (MV) was added to increase superoxide stress (Korbashi et al., 1986) and Lux activity was assayed after 3 h induction. Two millilitres of culture was dispensed into a 3 ml spectrofluorometer cuvette and 0.1 vol, pure decanal was added. Maximum light production was measured as emission during mixing (aeration) at 200 r.p.m. for 200 s in a Hitachi F-2000 spectrofluorometer. Lux activity was calculated as the difference between the maximum and the minimum light intensity (observed when stirring was turned off). Lux specific activity was calculated as intensity per c.f.u.

**DNA isolation and cloning.** Chromosomal DNA was extracted from A. vinelandii F196 following the procedure described by Robson et al. (1984). The DNA was partially digested with Sau3A under conditions optimized to generate fragments of approximately 23 kb (Sambrook et al., 1989). The digested DNA was ligated into the cosmid vector pLAFR3 and packaged using the Gigapack II Gold kit (Stratagene). The cosmid library was amplified in E. coli VCS257 and clones
containing Tn5luxAB were isolated on LB-Kan medium. The KanR clones were then screened for iron-repressible Lux activity by Petri plate contact printing (Sevinc & Page, 1992). Standard procedures were used for cloning, subcloning and restriction mapping (Sambrook et al., 1989).

**Southern hybridization.** DNA samples were transferred to 0.45 µm Hybond-N nylon membrane (Amersham Life Sciences) by the method of Southern (1975). The probe for luxA was a random primer labelled 330 bp PsrI fragment from within the luxA gene of pTn5luxAB (Feinberg & Vogelstein, 1983; Mehrotra, 1997).

**DNA sequencing.** Clone pAS40 was used as the template for double-stranded sequencing starting with primer WPPI (5′-CGTTGTAGATGGTGACG-3′), which was complementary to luxA sequence. Sequence downstream of the transposon was obtained, starting with primer WP107 (5′-GTCAGATCCTGGAAAACGG-3′), which was complementary to the right border of Tn5 (Auserwald et al., 1981). The sequence was determined using T7 Sequenase and Thermo Sequenase kits (Amersham Pharmacia Biotech).

**DNA: Sox binding assays.** The Sox protein of *A. vinelandii* was detected in the DNA-binding assay described by Yannone & Burgess (1997, 1998) using 15 µg CFX protein per reaction. The Sox-binding site of the ferredoxin:NADPH reductase gene (fpr) was synthesised as a double-stranded 50-mer oligonucleotide (Yannone & Burgess, 1998) and was used as a positive control. The Sox-boxes identified in this study were synthesised as double-stranded 50-mer oligonucleotides and end-labelled by standard procedure: 5′-TGACGCTAGCATTTACATACTGAGGGCCAGTGCCCTTCCCTAAGT-3′ (Box 1) and 5′-TTCCGATTGGCAGATTGGTCCGAGATTCTGCTCTCCAGAATGTGATAA-3′ (Box 2).

**DNA: Fur binding assays.** The binding of the *E. coli* Fur protein (a gift from Dr J. Neilands, University of California at Berkeley) to iron-box sequences was carried out as described by de Lorenzo et al. (1988), using Mn²⁺ as a corepressor. A 250 bp target containing the csbC promoter region was generated by PCR, using the oligonucleotide primers: WPJ21 (5′-ctacgctgacGCTCTGAGGGAAGGATGATG-3′) and WPJ22 (5′-ctctacgctgacGCTCTGAGGGAAGGATGATG-3′), which contained SalI (WPJ21) and BglII (WPJ22) restriction sites (lower case), to facilitate ligation in pQF50, generating pAS50. The PCR reaction mixture consisted of 10 µl 10 × Stemke buffer (0.7 M Tris, pH 8.8, 40 mM MgCl₂, 1% Triton X-100, 1 mg BSA ml⁻¹), 10 µl dNTPs (1 mM), approximately 30 pmol each of the primers, 20 ng pAS40 template and 5 µl Taq polymerase (diluted 1:10 in 1 × strength Stemke buffer) and deionized water (total volume 100 µl). The PCR program (Minicycley, MJ Research) was: 5 min denaturation at 95 °C, followed by 30 cycles of 30 s denaturing step at 95 °C, 30 s annealing at 52 °C and 1 min extension at 72 °C, and finally one 5 min extension at 72 °C. The Fur-binding site of the aerobactin (icsA) operator was excised as a 250 bp EcoRI–PstI fragment of pCON6 and was used as a positive control (de Lorenzo et al., 1988).

**Determination of Fur protein by Western blotting.** Cytoplasmic extracts were prepared from exponential-phase cultures (Maniatis et al., 1982) and the proteins were resolved by SDS-15% PAGE (Page & Huyer, 1984). Molecular mass was estimated using prestained standards from New England BioLabs. Proteins were transferred to a nylon membrane, which was then blocked in 1 × Blotto [10 mM Tris/HCl, pH 8.0, containing 0.5 M NaCl (TBS), 5% skim milk, and 0.02% sodium azide] for 30 min. A 1:500 dilution of polyclonal rabbit anti-Pseudomonas aeruginosa Fur antiserum (a gift from M. Basil, University of Colorado) was added and incubated with shaking overnight (Prince et al., 1991). The blot was washed with TBS, and Fur was localized by incubating with secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, 1:1000 in TBS) for 3 h at room temperature, followed by development with TBS containing 0.5 mg ml⁻¹ chloronaphthol in methanol and 0.02% H₂O₂.

**Isolation of RNA; Northern and dot-blot hybridization.** *A. vinelandii* and *E. coli* cultures were harvested in exponential phase for RNA extraction using a modified hot-phenol procedure (Frost et al., 1989; Miller, 1972). RNA (10 µg in 37 µl distilled H₂O) was denatured with 2.7 µl freshly deionized 6 M glyoxal, 8 µl DMSO and 16 µl 100 mM sodium phosphate buffer (pH 7.0) and incubated at 50 °C for 1 h. The cooled samples were loaded onto a 1% agarose gel (in 10 mM sodium phosphate buffer, pH 7.0) and separated by electrophoresis at 55–60 V for at least 4 h. In dot-blot analysis, each RNA sample (5 µg in deionized water) was denatured and loaded into the dot-blot well (Hybri-Dot manifold, BRL) as described by Thomas (1980). Northern hybridization was conducted at 60 °C (Sambrook et al., 1989). The probe used to detect the csbC transcript was a 310 bp NotI–PstI fragment cut from pAS310 and labelled using the random primer method. The probe used to determine even RNA loading was a universal oligonucleotide for the 16S rRNA gene (a gift from G. W. Stemke, University of Alberta).

**Primer extension.** Primer extension mapping was done as described by Sambrook et al. (1989), using end-labelled primer

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**Table 1. Cloning vectors and plasmid constructs**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>pLAFR3</td>
<td>Cosmid vector (TetR)</td>
<td>Staskawicz et al. (1987)</td>
</tr>
<tr>
<td>pK184</td>
<td>Low-copy-number cloning vector (KanR)</td>
<td>Jobling &amp; Holmes (1990)</td>
</tr>
<tr>
<td>pBluescript KS +</td>
<td>High-copy-number cloning vector ( AmpR)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pUC1919</td>
<td>High-copy-number cloning vector ( AmpR)</td>
<td>Vieira &amp; Messing (1987)</td>
</tr>
<tr>
<td>pQF50</td>
<td>Promoter-probe vector ( AmpR)</td>
<td>Farinha &amp; Kropinski (1990)</td>
</tr>
<tr>
<td>pMH15</td>
<td>E. coli fur gene cloned into pACYC184</td>
<td>Hankte (1984)</td>
</tr>
<tr>
<td>pAS1</td>
<td>23 kb Sau3A fragment of <em>A. vinelandii</em> F196 DNA cloned into pLAFR3</td>
<td>This study</td>
</tr>
<tr>
<td>pAS40</td>
<td>11.5 kb SalI fragment from pAS1 cloned into pUC1919</td>
<td>This study</td>
</tr>
<tr>
<td>pAS50</td>
<td>250 bp PCR csbC promoter fragment cloned into pQF50</td>
<td>This study</td>
</tr>
<tr>
<td>pAS310</td>
<td>310 bp NotI–PstI csbC fragment in pBluescript KS +</td>
<td>This study</td>
</tr>
<tr>
<td>pCON6</td>
<td>E. coli icsA cloned into pCON5</td>
<td>de Lorenzo et al. (1988)</td>
</tr>
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</table>
and 50 µg total RNA per reaction. RNA was denatured at 85 °C for 10 min, cooled to 45 °C, and hybridized with radiolabelled antisense primer at 45 °C for 1 h [3 × hybridization buffer, 0.5 µl (17 units) RNA guard]. Extension reactions were done with 25 units AMV reverse transcriptase (Boehringer Mannheim) at 42 °C for 1 h. The oligonucleotide WJP18 (5’-GGTAATGGGGTAACGAC-3’) was used as the primer, as it was approximately 140 bp from the putative 5’ start of cspC.

RESULTS
Iron-limited cells grow under oxygen-stress conditions

Previous studies showed that iron-limited (1 µM Fe³⁺) A. vinelandii strain UW cells had very low SOD specific activity, while iron-sufficient (75 µM Fe³⁺) cells had much greater SOD activity (Cornish & Page, 1998). When a wider range of iron-limited growth conditions was examined, it was found that the repression of catecholate siderophore synthesis coincided with the restoration of SOD activity (Fig. 1). Azotobactin was present in 1 µM Fe³⁺ medium [0.725 A₅₅₀ units (mg protein)⁻¹] and was completely repressed at 3 µM Fe³⁺, while catecholate siderophore production was not repressed until 10 µM Fe³⁺. SOD activity in these cells increased almost 50-fold from 1 to 10 µM Fe³⁺ (Fig. 1), then slowly increased in specific activity to 23 units (mg protein)⁻¹ at 50 µM Fe³⁺.

Since there is only a single iron-containing SOD in A. vinelandii (Cornish & Page, 1998), restored SOD activity indicated that more iron was available in the cytoplasm for SOD synthesis. Thus, conditions for intracellular hydroxyl radical generation through the Fenton reaction most likely existed at iron concentrations below 10 µM Fe³⁺, where intracellular iron was available, but SOD activity was low.

**Fig. 1.** SOD activity and catecholate siderophore production by A. vinelandii. The cells were grown overnight in Burk’s medium containing different iron concentrations. Cell extracts were assayed for SOD specific activity (○) and culture fluids were examined for catecholate siderophore production (●).

Iron-regulated control of Lux activity. Cultures were spotted onto iron-sufficient (+Fe) or iron-deficient (−Fe) agar medium and incubated overnight. Lux activity from strains (a) F196, (b) VCS257(pAS1), (c) VCS257(pAS1, pMH15) and (d) UW(pAS1) was recorded by exposure of X-ray film (Petri plate contact printing).

Cloning and sequencing the iron-repressible promoter (IRP) of strain F196

A. vinelandii strain F196 was used to further examine the control of catecholate siderophore synthesis. Strain F196 is a catecholate-minus mutant, which was generated previously by mutation of strain UW with Tn5luxAB (Sevinc & Page, 1992) so that Lux activity was regulated by iron availability (Fig. 2a). To isolate the IRP region of strain F196, a genomic library was prepared and a KanR clone, pAS1, was identified. However, Lux activity was poorly regulated by Fe³⁺ in E. coli VCS257(pAS1) (Fig. 2b). Iron-repressible Lux activity was restored when E. coli VCS257(pAS1) was transformed with pMH15, which encoded the E. coli fur gene on a plasmid with a copy number of 10–12 (Fig. 2c). Iron-repressible Lux activity was also observed when pAS1 was transformed into A. vinelandii UW (Fig. 2d) and into the Fur-overproducing E. coli strain HB101(pMH15) (data not shown). Therefore it was concluded that the IRP region was contained in pAS1 and that difficulties in observing Lux regulation in E. coli were due to repressor (Fur) dilution.

The restriction endonucleases BglII and SalI, which are known not to have sites in Tn5luxAB (Mehrotra, 1997), were used to digest pAS1, and the DNA fragments containing luxA were identified by Southern hybridization. An approximately 11.5 kb SalI fragment and a 10 kb BglII–SalI fragment of insert DNA were found to hybridize with luxA. Similar hybridizing bands were seen in similarly digested genomic DNA of strain F196. The fragments from pAS1 were subcloned into the low-copy-number vector pK184 so that iron-repressible Lux activity would be easier to assess. The clones were Lux⁺, indicating that the luxAB genes were contained within each fragment. Since pK184 encodes KanR, each fragment was cloned into pUC119 and was shown to also express KanR from Tn5. Because we wanted to obtain...
The 11 kb SalI fragment cloned in pUC119 (pAS40) was used as the template for sequencing. A total of 874 bp of double-stranded sequence upstream of the transposon and 489 bp of sequence downstream of the transposon were obtained. The sequence was analysed using FramePlot version 2.1 (Bibb et al., 1984) and two ORFs with opposite orientation were detected (Fig. 3). The complete sequence of the upstream ORF (orfA) is currently being determined. The 1084 bp sequence of the downstream ORF was subjected to a homology search, using the Gapped BLAST GenBank program version 2.0 (Altschul et al., 1997). There was an extremely high probability ($P = 3 \times 10^{-65}$) that the downstream ORF is an isochorismate synthase gene. It shared 44% identity and 55% similarity at the amino acid level with the isochorismate synthase gene from *Bacillus subtilis* and shared significant homology at the amino acid level with the corresponding genes from *Pseudomonas fluorescens*, *Mycobacterium tuberculosis*, *Vibrio cholerae*, *Aeromonas hydrophila* and *E. coli*. The gene encoding the isochorismate synthase of *A. vinelandii* (GenBank accession no. AF238500) was named *csbC*, for catecholate siderophore biosynthesis C, in keeping with the *E. coli* lettering system, where C denotes isochorismate synthase.

A putative promoter, characteristic of a typical *E. coli* $\sigma^{32}$ promoter (Lewin, 1994) and a putative iron-box, having 14 out of 19 bp identical to the *E. coli* iron-box consensus sequence (de Lorenzo et al., 1987), was identified overlapping the $-35$ region of the *csbC* promoter (Fig. 3). The upstream gene (orfA) appeared to be controlled from a divergent $-10$ region of *E. coli* Fur protein. Anti-*P. aeruginosa* Fur antiserum was reacted with 6 µl cell extract proteins from *A. vinelandii* (lane 1); 15 µl cell extract of Fur$^-$ *E. coli* BN4020 (lane 2) and Fur$^+$ *E. coli* BN402 (lane 3) and 0.5 µg purified *E. coli* Fur protein (lane 4).

Fig. 4. Fur interaction with the *csbC* region. (a) Gel shift assay (with Mn$^{2+}$ corepressor) using the 250 bp *A. vinelandii* *csbC* promoter fragment and increasing amounts of the *E. coli* Fur protein in lanes 1–7: 0, 25, 75, 150, 300, 500 and 700 nM. Arrows show initial target shifts. Lanes 8–10: competition assay with 300 nM Fur protein and 10× (lane 8) or 50× (lane 9) *csbC* promoter fragment as specific competitor, or with 2000× poly(dl-dC)-non-specific competitor (lane 10). (b) Western blot for the detection of the *A. vinelandii* Fur protein. Anti-*P. aeruginosa* Fur antiserum was reacted with 6 µl cell extract proteins from *A. vinelandii* (lane 1); 15 µl cell extract of Fur$^-$ *E. coli* BN4020 (lane 2) and Fur$^+$ *E. coli* BN402 (lane 3) and 0.5 µg purified *E. coli* Fur protein (lane 4).
that was 7 bp downstream of the proposed −10 sequence (Fig. 3, asterisk).

Northern analysis

The RNA transcript from csbC expressed in E. coli or in A. vinelandii appeared as a smear of hybridization, running through the length of the gel when examined by Northern analysis (data not shown). These results are typical of those obtained by Northern analysis of the ent operon of E. coli (T. Brickman, personal communication). The transcript was only detectable in iron-limited cells and the csbC transcript was completely repressed under iron sufficient (7–10 µM Fe⁺⁺) growth conditions. Exact transcript sizes were hard to determine, but the largest transcript was approximately 3·2 kb in strain F196 and approximately 9·5 kb in the wild-type strain UW. This difference in size was most likely due to the termination of transcription at the 3′ end of luxAB in strain F196. Since luxAB accounted for approximately 2·4 kb, and the sequence between the putative promoter and start of luxA was about 660 bp, the predicted truncated transcript in strain F196 was about 3060 bp, as observed. When the Northern blot was stripped and probed with a universal oligonucleotide for 16S rRNA, bands of approximately even intensity were seen in all lanes, indicating that absence of csbC product under iron-sufficient growth conditions was due to repression, not to problems in RNA isolation or loading (data not shown).

DNA binding by the E. coli Fur protein

In order to further investigate the possibility that a Fur-like protein regulated csbC, DNA-binding assays using purified E. coli Fur protein were done. Although it would have been better to use the A. vinelandii Fur protein in these assays, this product was not available. Use of the E. coli Fur protein seemed reasonable since Fur is highly conserved and the E. coli Fur will complement fur mutations in many bacteria (Achenbach & Yang, 1997). The DNA target was the 250 bp PCR fragment from pAS50 containing the putative promoters and iron-boxes of csbC and orfA (Fig. 4). A DNA shift was observed at ≥ 25 nM Fur (Fig. 4a, lane 2), and a second shift was detected at ≥ 500 nM Fur (Fig. 4a, lane 6). This was not unexpected, since there were two iron-boxes within the target DNA, with relatively high (csbC iron-box) or low (orfA iron-box) homology to the iron-box consensus sequence. When the corepressor Mn²⁺ was absent, the DNA shift was eliminated (data not shown). The assay was repeated in exactly the same way using a 250 bp EcoRI–PvuII fragment containing the E. coli aerobactin (iucA) promoter. In this positive control, a similar shift was observed only in the presence of Mn²⁺, at ≥ 50 nM Fur (data not shown).

To further confirm that the binding of E. coli Fur to the 250 bp PCR fragment was specific, a competition binding assay was done. The labelled target was mixed with a 10- to 50-fold excess of unlabelled 250 bp fragment as a specific competitor (Fig. 4a, lanes 8 and 9), or with excess poly(dI-dC) as a non-specific competitor (Fig. 4a, lane 10), prior to mixing with the binding buffer/Fur mixture. These assays show that this protein–DNA interaction was specific, as Fur binding was out-competed by excess specific competitor. However, Fur still bound to the labelled target DNA even in the presence of a 2000-fold excess of non-homologous DNA.

Cell extract of A. vinelandii strain UW contained a single protein that cross-reacted strongly with anti-P. aeruginosa Fur antiserum. This protein had a molecular mass of approximately 17 kDa, slightly larger than the Fur protein found in E. coli (Fig. 4b).

Regulation of csbC transcription by iron and oxidative stress

In order to determine if csbC transcription was also regulated by O₂ stress, cultures were grown for 22 h in different concentrations of iron citrate, with the aeration altered by varying the culture volume per flask. Increased aeration increased catecholate siderophore production and delayed the repressive effect of added Fe⁺⁺ (Fig. 5a). The transcription of csbC was repressed by lower
Methyl viologen increases csbC activity

Lux activity in strain F196 could be upregulated in response to methyl viologen (MV), an in vivo superoxide generator (Korbashi et al., 1986). In this assay, cells of strain F196 were grown under partially repressive conditions (5 μM Fe$^{3+}$) so that upregulation of Lux could be observed (Fig. 6). Lux activity was very low in the 5 μM Fe$^{3+}$ control (Fig. 6, no added MV) but was upregulated at least 400-fold after a 3 h incubation with 30 μM MV. However, when excess Fe$^{3+}$ had repressed Lux activity, it could not be induced by MV addition (data not shown).

DNA binding by a putative Sox protein

A DNA-binding protein with an affinity for Sox-boxes was identified in cell extracts of strain UW (Fig. 7a) using the gel-retardation assay conditions described by Yannone & Burgess (1997). It was apparent from these assays that Box 1 had a higher affinity for the binding protein than Box 2. Also, the binding protein was constitutive and not regulated by iron availability. When a 50-mer oligonucleotide containing the fpr Sox-box was similarly examined, the magnitude of the gel shift was identical to that obtained with Box 1 (Fig. 7b). Also included were cell extracts from iron-limited A. vinelandii strain LM100, which is defective in ferredoxin I (FdI) formation (Morgan et al., 1988). It has been

concentrations of ferric citrate under low aeration conditions than under high aeration conditions (Fig. 5b), suggesting that control by Fur and a Sox-element may be in competition with each other.
speculated that FdI modulates the activity of the A. vinelandii Sox protein (Yannone & Burgess, 1997), but according to these results FdI is not required for DNA binding-activity (Fig. 7b).

**DISCUSSION**

In this study we have isolated the A. vinelandii gene for isochorismate synthase, which is responsible for the first step in catecholate siderophore biosynthesis (Crosa, 1989). This gene (csbC) is likely to be part of an operon, since an RNA transcript of about 9.5 kb was identified by Northern analysis. Operon structure is very probable, since the first steps in catecholate siderophore synthesis are highly conserved in different bacteria and the corresponding genes are organized together (Massad et al., 1994). Furthermore, the observation that feeding 2,3-dihydroxybenzoic acid to iron-limited strain F196 did not restore catecholate siderophore production (Sevinc & Page, 1992) suggests that the Tn5 insertion in csbC has a polar effect on downstream genes required for siderophore assembly.

The regulatory region upstream of csbC is reminiscent of the divergent promoter to the E. coli fepB–entC region (Ozenberger et al., 1987), where the –35 sequence of the entC promoter is separated from the –35 sequence of fepB by 31 bp. In A. vinelandii the –35 sequence of csbC is separated by 30 bp from the –35 sequence of the divergent promoter of orfA, which however does not share any homology with fepB. Our preliminary results on the sequencing of this ORF (unpublished work in progress) indicate some homology to antibiotic efflux transport proteins. One such protein is a chloramphenicol-resistance determinant that encodes a putative transmembrane pump in Streptomyces lividans (Dittrich et al., 1991). This protein shares 20% homology with the integral membrane protein FhuB involved with the ferric-hydroxamate siderophore uptake system in E. coli. It is possible that the orfA product is involved in catecholate siderophore transport and may be coordinately regulated with catecholate biosynthesis.

It is clear that csbC transcription is negatively regulated by iron. The transcription of a csbC::luxAB fusion is negatively affected by E. coli Fur (introduced on pMH15) and E. coli Fur binds to the iron-boxes in the orfA–csbC intergenic region. An A. vinelandii Fur homologue exists, but we have been unable to obtain this product to use in these binding assays. The csbC iron-box has a good degree of homology with the 19 bp Fur binding consensus sequence (de Lorenzo et al., 1987) and is somewhat palindromic (Fig. 8a). The orfA iron-box, on the other hand, has a lower homology with the 19 bp consensus sequence (Fig. 8a). In a new interpretation of the iron-box (Escolar et al., 1999), it has been suggested that the consensus is comprised of at least three 6 bp repeats (Fig. 8b). The thymines present in the AT-AT motif are involved in binding to the repressor (Dalet et al., 1999). In this analysis, the csbC iron-box has three contiguous repeats with at least an AT-T motif. The orfA iron-box has weaker homology, with only two contiguous repeats with an AT-T motif (Fig. 8b). These iron-boxes should have different affinities for Fur binding. Indeed, two shifts in the mobility of the orfA–csbC intergenic region were observed with the addition of Fur-Mn²⁺ (Fig. 4a). The highest affinity observed was like that of the ituA iron-box of E. coli, which suggests that csbC transcription should be tightly controlled by iron availability, if the only regulatory feature was Fur. However, csbC transcription continues at iron concentrations greater than 3 μM but less than 10 μM. At 3 μM iron, azotobactin synthesis is repressed (Page & von Tigerstrom, 1988), indicating that an active Fur complex exists in the cytoplasm for repression of siderophore synthesis.

Thus, based on previous speculation (Cornish & Page, 1998), we explored the possibility that a superoxide stress (Sox) regulator may also be involved in the control of catecholate siderophore synthesis. Indeed, repression of csbC transcription by iron was delayed in aerated cultures and the activity of a csbC::luxAB fusion was increased in cells treated with MV. Two putative Sox-boxes were identified in the csbC intergenic region, using the long consensus sequence proposed by Fawcett et al. (1993). Symbols: ●, identity to E. coli consensus sequences; ○, identity to fpr Sox-box; –, altered sequence; underlining, palindromic regions. Nucleic acid codes: R = G or A, W = A or T; Y = T or C; N = any residue.

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**Fig. 8.** (a, b) Comparison of A. vinelandii and E. coli Fur and Sox binding sites: comparison of the csbC and orfA iron boxes with (a) the E. coli 19 bp palindromic consensus sequence (de Lorenzo et al., 1987) and (b) the E. coli 6 bp repeat consensus sequence (Escolar et al., 1999). (c) Comparison of A. vinelandii and E. coli consensus Sox-box sequences (Fawcett & Wolf, 1994). Mutants of the fpr Sox-box (S+P− and S−P+) are from Regnstrom et al. (1999)."
& Wolf (1994) (Fig. 8c). The only known Sox-box in A. vinelandii is the one found upstream of the fpr promoter (Yannone & Burgess, 1997, 1998). This sequence has a palindromic structure (Fig. 8c) that was thought to be important in Sox protein binding (Yannone & Burgess, 1997). However, elimination of the palindrome (S+P in Fig. 8c) does not adversely affect protein binding, while mutations in the motif AYNGCAY (S−P+ in Fig. 8c) does eliminate protein binding (Regnstrom et al., 1999). Therefore, only the first 17 bp may be important in Sox protein binding in A. vinelandii. Only the csbC Box 1 had an intact GCAY motif and had good identity (10/17 bp) to the fpr Sox-box (Fig. 8c).

However, the binding of the presumed Sox protein to Box 1 cannot be considered equivalent to its binding to the fpr Sox-box. The GCAY motif of Box 1 is located at the —10 region of the csbC promoter (Fig. 3). A protein binding in this region could be a class II activator (Ishihama, 1992). The best example of such an activator is the MerR protein, which controls the mercury-resistance locus of Tn501 (Summers, 1992). This protein, which is related to the superoxide stress regulator SoxR (Ishihama, 1992), acts as a repressor until activated by mercuric ions. A conformational change results in underwinding of the DNA at the MerR target, allowing mercuric ions. A conformational change results in underwinding of the DNA at the MerR target, allowing

REFERENCES


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