The Escherichia coli dsbA gene is partly transcribed from the promoter of a weakly expressed upstream gene

Pascal Belin and Paul Louis Boquet

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The dsbA gene of Escherichia coli encodes a periplasmic enzyme which catalyses disulfide bond formation. Analysis of its surrounding DNA region showed that it is preceded by an open reading frame, orfA, of 984 nucleotides. The intergenic region (19 nucleotides) carries no typical transcription termination signals. dsbA is transcribed from two promoters, the first (P1) lies in the distal part of orfA, and the second (P2) just upstream from orfA. Using a plasmid-borne dsbA::TnphoA fusion and an orfA::Ω insertion, each promoter was shown to contribute equally to dsbA transcription. The disruption of the single chromosomal copy of orfA by Ω more drastically reduced the amount of DsbA in the periplasmic space. Such a reduction of the DsbA pool, however, did not change the activities of the AppA, Agp and PhoA periplasmic phosphatases, which all require disulfide bond formation, even when the enzymes were produced from multicopy recombinant plasmids. Thus, in a wild-type strain, DsbA is far from being in limiting amounts for physiological requirements. The orfA gene product was identified as a weakly expressed 39 kDa cytoplasmic protein, but it is not involved in the overall mechanism of disulfide bond formation.

**Keywords:** Escherichia coli, dsbA gene, transcriptional organization, internal promoter, disulfide bond formation

INTRODUCTION

The folding of proteins in vivo takes place very rapidly because it is assisted by a variety of factors whose functions extend from preventing improper folding and aggregation to catalysing rate-limiting steps such as proline cis/trans isomerization and disulfide bond formation in secreted proteins. These factors are widely distributed among the different organisms, and most of the cellular compartments have their own representative (Gething & Sambrook, 1992). Until recently, little was known about the folding of bacterial secreted proteins, although a disulfide isomerase activity had been detected in the periplasmic space of Escherichia coli (Barth et al., 1988). A gene, dsbA, encoding a periplasmic protein involved in this process, has been identified at 87 min on the E. coli linkage map by several groups (Bardwell et al., 1991; Kamitani et al., 1992; Belin et al., 1994) and shown to exist also in other micro-organisms (Peek & Taylor, 1992; Yu et al., 1992; Tomb, 1992). The DsbA protein contains a Cys-X-X-Cys motif common to several thiol-disulfide oxido-reductases and is able to catalyse the formation of disulfide bridges in vitro (Akiyama et al., 1992). The oxidized form has an intrachain disulfide bridge of high instability and transfers this oxidized state to cysteine residues of substrate proteins very efficiently, the DsbA protein being reduced in the process (Zapun et al., 1993). The amount of DsbA in the periplasmic space has been estimated at around 850 molecules per cell (Akiyama et al., 1992). However, periplasmic proteins containing disulfide bonds such as AppA and Agp (acid phosphatases) or PhoA (alkaline phosphatase) can be over-produced from recombinant plasmids in their mature state, to levels of 60–80% of total periplasmic proteins. This implies a very efficient reoxidation of reduced DsbA which takes place even under anaerobic conditions (Belin et al., 1994). Another gene (dsbB or dsbX), whose inactivation leads to a phenotype very similar to that of dsbA mutants, has been identified at 26 min on the E. coli linkage map (Bardwell et al., 1993;


<table>
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<tr>
<th>Strain or plasmid</th>
<th>Description of the genotype</th>
<th>Source</th>
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<tr>
<td><strong>E. coli</strong></td>
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<tr>
<td>CC118</td>
<td>F^* Δ(ara leu)7697 ΔlacX74 ΔphoA20 galE galK thi rpsE rpoB argE(Am) recA1</td>
<td>C. Manoil (University of Washington, Seattle)</td>
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<td>CSR603</td>
<td>F^* argE3 ara-14 galK2 leuB manl phr-1 proA2 recA1 rpsL31 supE44 thi thr-1 uraA6 xyl-5</td>
<td>E. Dassa (Pasteur Institute, Paris)</td>
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<tr>
<td><strong>K10</strong></td>
<td>HfrC relA tonA22 pit-10</td>
<td>B. Bachmann (Yale University, New Haven)</td>
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<tr>
<td>SBS2227</td>
<td>Same as K10, but orfA::Ω</td>
<td>This study</td>
</tr>
<tr>
<td>SBS2171</td>
<td>HfrC relA pit-10 tonA22 thi polA12(ts) Tn10-64 (Tn10 90% cotransducible with polA)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pHSG575</td>
<td>Cm'</td>
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<tr>
<td>pHP45O</td>
<td>Amp' Str' Spe'</td>
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<td>pBR322</td>
<td>Amp' Tet'</td>
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<td>pPB21132</td>
<td>Amp' appBA</td>
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<td>pEP1376</td>
<td>Amp' qgg</td>
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<tr>
<td>pPB2142</td>
<td>Cm' dsbA (HindIII–HindIII insert into the HindIII site of pHSG575)</td>
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<tr>
<td>pPB2144</td>
<td>Cm' mob orfA (EcoRI–PstI insert into the EcoRI–PstI sites of pHSG575)</td>
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<td>pPB2181</td>
<td>Amp' dsbA !Alf(Oc)</td>
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<td>pPB2212</td>
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<td>Amp' dsbA (a 0.9 kb ΔHindIII deletion on pPB2354)</td>
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<td>pPB2394</td>
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<td>Amp' orfA' dsbA::TnphoA (BamHI digestion, fill-in and ligation on pPB2354)</td>
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* Sites that have been filled with T4 DNA polymerase.
Dailey, 1993; Belin & Boquet, 1993; Missiakas et al., 1993). Nucleotide sequence analysis showed that the encoded protein has the characteristics of an integral membrane protein (Bardwell et al., 1993; Missiakas et al., 1993). Inactivation of dsbB is rescued by the addition to the culture medium of oxidized glutathione (Bardwell et al., 1993; Belin & Boquet, 1993) or cystine (Bardwell et al., 1993; Dailey & Berg, 1993), strongly suggesting that the DsbB protein is involved in DsbA reoxidation.

At present there is no information available about possible regulation of DsbA production as a function of the need for this factor or in response to physiological changes. Recently, Plunkett et al. (1993) published the E. coli genome nucleotide sequence from 87-2 min to 89-2 min. The dsbA gene, included in this region, was found to be preceded by an open reading frame, referred to as o328. No sequences resembling putative terminators of transcription can be distinguished upstream of dsbA (Kamitani et al., 1992; Plunkett et al., 1993). Furthermore, Plunkett et al. (1993) identified, by computer analysis, two putative promoters upstream of o328 which could transcribe o328 and dsbA simultaneously. The aim of this study was to analyse dsbA transcription and expression. We have identified the o328 gene product, and analysed the consequences of its disruption on growth, DsbA production and disulfide bond formation.

**METHODS**

**Strains, growth conditions and genetic techniques.** All the strains used in this study are derivatives of *Escherichia coli* K12 and are described in Table 1. Transductions were made with phage P1<sub>mr</sub> according to Miller (1972). Cells were grown in TYE medium (bacto tryptone, 10 g l<sup>-1</sup>; yeast extract, 5 g l<sup>-1</sup>; and NaCl, 8 g l<sup>-1</sup>, pH 7.5) as a rich medium. Ampicillin (Amp) was added to a final concentration of 200 μg ml<sup>-1</sup> and chloramphenicol (Cm) at 10 μg ml<sup>-1</sup>, unless otherwise stated. Streptomycin (Str), spectinomycin (Spc) and tetracycline (Tet) were added as indicated in the text. The induction of the P<sub>lac</sub> promoter was obtained with IPTG (2 mM).

**Plasmids and DNA manipulations.** The plasmids used in this study are listed in Table 1. Plasmid DNA manipulations were performed according to Sambrook et al. (1989). The Ω interposon was isolated from plasmid pH645Ω constructed by Prentki & Krisch (1984).

**Genomic DNA extraction and analysis by PCR.** Genomic DNA was isolated according to Silhavy et al. (1984). The analysis of genomic DNA by PCR was performed using oligonucleotide 5′-GATTCAATTAAAGTCTGACGTT-3′ which is complementary to nucleotide position 272-292 of orfA (o328), and oligonucleotide 5′-CGGCCTGTTAAATGGAGTAAAA-3′, which corresponds to a nucleotide sequence lying just before the HindIII site 70 nucleotides upstream from the initiation codon of orfA. PCR amplification was carried out in 100 μl volumes containing 1 μg DNA and 10 ng of each oligonucleotide in standard buffer supplemented with 10% (v/v) DMSO (Khalil et al., 1992). Reactions were heated to 95 °C for 5 min, and 2.5 U Taq DNA polymerase was added to each reaction. Thirty cycles were performed as follows: melting at 92 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min (extension 5 s at each cycle). After a 10 min extension at 72 °C, 10 μl of each reaction was analysed on 1% (w/v) agarose gel (Sambrook et al., 1989).

**DNA sequencing and sequence analysis.** Nucleotide sequence determinations were made on both strands of plasmid DNA using the Pharmacia T7 sequencing kit. Denaturation of plasmid DNA and sequence reactions were performed according to the instructions of the manufacturer. The sequence data were analysed with the DNA Strider 1.2 program (Marc, 1988). Homologies were searched for in the NBRF databases (release number 38) by using the BISANCE facilities (Dessen et al., 1990).
Fig. 2. For legend see facing page.
was rinsed with 70% (v/v) ethanol, vacuum-dried and dissolved in distilled water. RNA concentration was determined by absorbance at 260 nm and the RNA was stored at −20 °C.

For reverse transcriptase analysis, the oligonucleotides 5'-CTTCAACTGATCGACGGGTCG3', which is complementary to position 146–169 of the dsbA gene, and 5'-GAGATCCATGCTCAACAGAGGTCATGAT3', which is complementary to position 46–76 of orfA, were used. The oligomers were 5'-end-labelled by using polynucleotide kinase and [γ-32P]ATP. A molar excess of oligonucleotides was annealed with a different amount of RNA according to Sambrook et al. (1989). Primer extension analysis was performed according to Sambrook et al. (1989). The products of the primer extensions were analysed by electrophoresis on an 8% (w/v) polyacrylamide–urea gel and autoradiography.

Analysis of proteins. The enzymic specific activities of pH 2.5 acid phosphatase, glucose-1-phosphatase and alkaline phosphatase were measured and expressed as reported previously (Dassa et al., 1982; Pradel & Boquet, 1988; Belin et al., 1994). Periplasmic extracts were prepared by an osmotic shock procedure (Neu & Heppel, 1965) and were submitted to analysis by SDS-PAGE according to Laemmli (1970) and silver-stained (Blum et al., 1987).

RESULTS
Nucleotide sequence analysis and genetic organization of the dsbA region

The cloning of the dsbA gene with phagemid MudI1117 has been reported previously and the recombinant carriers large fragments lying upstream and downstream from dsbA (Belin et al., 1994). The dsbA upstream region was subcloned by inserting a 5.6 kb EcoRI–PstI fragment between the EcoRI and PstI sites of the vector pHSG575 (Takeshita et al., 1987), yielding plasmid pPB2144 (Fig. 1). The dsbA downstream region was also subcloned on pHSG575 yielding plasmid pPB2142, also described (Fig. 1; Belin et al., 1994).

Nucleotide sequence analysis was performed on both DNA strands and extended from a BamHI site lying 2.1 kb upstream of the dsbA ATG initiation codon to a HindIII site located 2.3 kb downstream from the same triplet (Fig. 1). This sequence corresponds to the fragment located between positions 3664 and 3660 of Kohara’s map (Kohara et al., 1987).

The dsbA gene was found to be followed by a 1428 nucleotide open reading frame orfB and encoding a putative polypeptide of 476 amino acid residues. The difference in length with 0490 described by Plunkett et al. (1993) results from the choice of the start codon. Here we ascribed ATG as the initiation codon for orfB, rather than the GTG triplet found 42 nucleotides upstream. dsbA and orfB were found to be separated by sequences similar to a typical r-independent terminator of transcription, indicating that they very likely belong to different transcrip-

tional units. The orfB open reading frame is followed by a structure which could form an RNA hairpin, and could correspond to a terminator of transcription.

The sequence immediately upstream from dsbA contains another open reading frame, orfA (Fig. 2). It is 984 nucleotides long and terminates only 19 nucleotides upstream from the ATG initiation codon of dsbA. This sequence potentially encodes a polypeptide of 328 amino acid residues with a calculated molecular mass of 38096 Da and with a codon adaptation index (CAI) of 0.27 (Sharp & Li, 1987). The nucleotide sequence of orfA was found to be identical to 0328 described by Plunkett et al. (1993). No significant homology with any of the proteins in the NBRF database were found. As noted by Plunkett et al. (1993), no typical terminator of transcription can be found between orfA and dsbA.

The mob gene lies 416 nucleotides upstream from the orfA initiation codon and is transcribed in the opposite direction (Plunkett et al., 1993). The region between the mob gene and orfA contains a short open reading frame named o89 by Plunkett and coworkers. It is also possible to see a small open reading frame, orfC, on the other DNA strand of this intergenic region, preceding the mob gene and transcribed in the same direction (Fig. 1).

We found some differences between the present nucleotide sequence and that described by Plunkett et al. (1993). Firstly, their sequence reads TGATAATCTCCT 4024, while our sequence reads TGATATC: the superinumersary A (underlined) would eliminate the EcoRV site (bold type) found 33 nucleotides before orfA. We found that this restriction site actually exists, as also described by Kohara et al. (1987). Secondly, three differences were found in the putative dsbA transcription terminator. However, none of the other authors who have sequenced this region (Bardwell et al., 1991; Kamitani et al., 1992) obtained exactly the same nucleotide sequence. Thirdly, individual nucleotide substitutions were found within orfB (0490), which did not change the reading frame.

Identification of the orfA gene product

To determine whether orfA was actually expressed as a gene, maxicell analysis was performed with strain CSR603 harbouring either plasmid pPB2234, which carries orfA and dsbA on a SmaI–SspI fragment inserted between the EcoRI and NruI sites of pBR322, or plasmid pPB2196, which contains a larger orfA upstream DNA region (Fig. 1). In addition to the DsbA protein (apparent molecular mass 24 kDa, Bardwell et al., 1991) and β-lactamase (two bands around 30 kDa, Sancar et al., 1979), the plasmids were found to encode a polypeptide with an apparent molecular mass of 39 kDa. A partial deletion of orfA was constructed on plasmid pPB2234 between both HindIII sites, resulting in plasmid pPB2366. This plasmid did not

Fig. 2. Nucleotide sequence of the orfA gene and its flanking regions. The orfA gene and the beginning of the dsbA gene are translated into protein. SD indicates the putative Shine–Dalgarno sequences. The origins of transcription are indicated by ●. The deduced –10 and –35 regions are overlined. The SmaI, EcoRV and BamHI restriction sites are indicated.
produce the 39 kDa polypeptide in maxicells (Fig. 3). Therefore, orfA actually encodes this polypeptide. The absence of a typical signal peptide and of hydrophobic regions similar to membrane-spanning domains in the orfA amino acid sequence indicates that it is probably a cytoplasmic protein. Furthermore, we have obtained active TnlacZ fusions all along the orfA gene on plasmid pPB2234. After cellular fractionation of bacteria carrying such a fusion plasmid, the β-galactosidase activity was localized to the cytoplasmic fraction, as opposed to being membrane-associated (data not shown).

The products of the orfC genes carried by plasmid pPB2196 were not observed in our maxicell analyses.

Determination of the origins of transcription of dsbA and orfA

The interval between orfA and dsbA appears too small to contain a promoter region, and as previously noted by Kamitani et al. (1992), no typical promoter sequence can be identified unambiguously for dsbA. However, a dsbA mutation was complemented by plasmid pPB2212, which carries the dsbA gene on a HindIII–SspI fragment inserted between the HindIII and NruI sites of pBR322 (Fig. 1). Inserting a 2 kb Ω interposon which contains a StrR and SpcR gene flanked by transcription and translation termination signals, and multiple cloning sites (Prentki & Krisch, 1984) into the HindIII site of pPB2212 did not change its complementing activity as well as the amount of DsbA produced in the periplasmic space (data not shown). This rules out the possibility that, on pPB2212, dsbA could be transcribed from a pBR322 endogenous promoter. Consequently the HindIII–SspI fragment actually carries a promoter for dsbA, which very likely is located in the distal part of orfA.

The origins of transcription of dsbA and orfA were determined by primer extension analysis, using mRNA...
extracted from cells of strain CC118 harbouring pPB2196. mRNA (0.5 μg) was first hybridized with a 32P-labelled 32mer oligonucleotide complementary to nucleotide position 146–169 of the dsbA gene (see Methods). The length of the cDNAs obtained after the action of reverse transcriptase corresponded to a main initiation point at the T in position 2053 of our sequence (Fig. 4). The most probable −10 box (TACAAG) is located 8 nucleotides upstream from this point and shows 66% sequence identity with the −10 σ70-dependent consensus box. The predicted −35 box (TTTATA) is 16 nucleotides upstream of the −10 box and also shows 66% identity with the −35 σ70-dependent consensus sequence (Fig. 2). The most noteworthy feature of this promoter is its localization inside the terminal part of the orfA gene. Several closely linked initiation points of minor importance were also observed (Fig. 4), but they did not correspond to unequivocal promoter sequences.

The origin of transcription of orfA was determined with the same method using an oligonucleotide complementary to nucleotide position 46–76 of the orfA gene (see Methods). To obtain a weak signal, 25 μg of RNA was necessary. The orfA origin of transcription was found at the T in position 1067, 24 nucleotides upstream from the orfA initiation codon. The sequence TATCCT located 8 nucleotides upstream from the origin of transcription is predicted to be the −10 box (Fig. 2). The −35 box is more difficult to identify, and the most likely sequence is TGn upstream of the −10 box, a distance which is not typical for a σ70-dependent consensus promoter. One interesting feature of this promoter is the presence of the sequence TGn just before the −10 box. This upstream sequence, found in promoters having the major −10 consensus sequence, allows initiation without the help of the −35 region (Kumar et al., 1993). The characteristics of this promoter will be discussed below (see Discussion). Another interesting feature is the poor conservation of the Shine–Dalgarno (SD) sequence of orfA, for which only three successive nucleotides (GGA) match the known consensus (Shine & Dalgarno, 1974).

Can dsbA be transcribed from the orfA promoter?

The location of a dsbA-proximal promoter inside the orfA gene, the characterization of an orfA promoter and the lack of transcription termination signals between orfA
and dsbA raise the possibility of a dual promoter transcription of dsbA. If dsbA and orfA belong, at least partly, to the same transcriptional unit, the contribution of each promoter to DsbA expression remains to be evaluated. This can be done by inserting a transcription and translation termination element between the two promoters and evaluating DsbA expression. A convenient way would be to measure the expression of alkaline phosphatase (AP) produced from a orfA::TnphoA downstream fusion. One such fusion obtained by TnphoA insertion between nucleotides 79 and 80 of the orfA gene has already been described and is carried on plasmid pPB2354 (Belin et al., 1994). The resulting processed hybrid protein, which contains only seven amino acid residues of the amino-terminal part of DsbA, is stable in bacteria of strain CC118 containing each plasmid was measured. The first deletion extends from the Smal site to the EcoRV site (plasmid pPB2360) and the second from the EcoRV site to the BamHI site located within the orfA gene (plasmid pPB2394). In each case, the deletion promoted about a twofold reduction of expression of AP (Fig. 6), as previously observed with plasmid pPB2358. This result shows that the dsbA gene is actually transcribed from the orfA promoter, and confirms the position of the −10 box in this promoter around the EcoRV site, a result in good agreement with the primer extension experiment described above.

**Consequences of orfA disruption**

The orfA gene carried by plasmid pPB2216 was inactivated by insertion of the fragment into the unique BamHI site (pPB2217, see Fig. 1). The orfA::Ω construct was introduced into the chromosome by homologous recombination (Gay, 1984). Transformants of SBS2171 (polAΔ) carrying plasmid pPB2217 were selected by growth in the presence of Str and Spc (50 μg ml⁻¹ each). The plasmid was forced to integrate into the chromosome at 37 °C in serial cultures containing low levels of Str and Spc (10 μg ml⁻¹ each). Its return to the autonomous state by further growth at 30 °C in the presence of 200 μg Amp ml⁻¹ was followed by plating in the presence of Amp (200 μg ml⁻¹), and Str and Spc (50 μg ml⁻¹ each) to discriminate between clones carrying the original plasmid with Ω in the multicopy state (large colonies) and the recombinant clones carrying only a chromosomal copy of Ω (small colonies). Bacteria from a small colony were grown at 42 °C without antibiotic to eliminate the recombinant plasmid. A stock of phage P1 made on such a recombinant was used to transduce bacteria of strain K10 to Str⁺ Spc⁺ (10 μg ml⁻¹ each). A Tet⁺ Str⁺ Spc⁺ and polA⁺ transductant (SBS2227) was selected. Further transduction experiments made with this strain as donor showed that the Str⁺ Spc⁺ character was integrated very close to dsbA.

To test further for the presence of Ω in the orfA gene of SBS2227, this region of the chromosome from bacteria of strain K10 and SBS2227 was amplified by PCR, using oligonucleotides complementary to sequences lying on each side of the BamHI site in which Ω was inserted (see Methods). The PCR products found for both strains showed the expected sizes, i.e. a single band of about 0.5 kb for K10 and a single band of about 2.5 kb for SBS2227. The 2.5 kb PCR band obtained from SBS2227 yielded a 2 kb band when digested with BamHI (data not
Nucleotide sequence analysis indicated that the mutation in pPB2395. However, when the second com-
to try to avoid a block of its transcription from the P2 
ments were performed. Firstly, bacteria of strain SBS2227 
(orfA::52) was mutated only by frameshifting 
read frame was actually destroyed (data not shown). 
shown). This fragment, when religated into the BamHI 
site of pBR322, conferred resistance to Str and Spc. This 
eliminates the possibility that an intact orfA gene would 
lie in tandem close to orfA::Ω. Interrupting orfA by Ω on 
the chromosome of SBS2227 caused a drastic reduction in 
the amount of DsbA produced (Fig. 7a).

The ability of bacteria from strain SBS2227 to form 
disulfide bridges in DsbA-dependent periplasmic phos-
phatases was examined. The activities of pH 2.5 acid 
phosphatase (AppA), glucose-1-phosphatase (App) and 
alkaline phosphatase (PhoA) were the same in SBS2227 
and K10 (data not shown). Moreover, when bacteria of 
both strains were transformed with plasmids over-
producing the same phosphatases, namely pPB1132 for 
Sac (Boquet et al., 1987), pEP1376 for App (Pradel 
& Boquet, 1988) and pLIP5.0 for PhoA (φoA under 
the control of the Ptap promoter in the pACYC177 vector; 
L. Cattolico, this laboratory, unpublished), none of these 
activities were reduced in SBS2227 (data not shown). 
Consequently, under the conditions used, disulfide bond 
formation was not altered by the presence of Ω in orfA, 
showing that the orfA gene product itself is very likely 
not involved in this process.

To discover whether normal expression of dsbA would 
require an intact orfA gene product, two sets of experi-
ments were performed. Firstly, bacteria of strain SBS2227 
were transformed with plasmid pPB2144 which carries 
the entire orfA gene cloned into the vector pBR322. 
The amount of DsbA in the periplasmic space of the 
transformants was similar to that in SBS2227 (Fig. 7b). 
Secondly, the orfA gene carried by plasmid pPB2354 
(orfA dsbA::TnphoA) was mutated only by frameshifting 
to try to avoid a block of its transcription from the P2 
promoter. The plasmid was cut with BamHI and the gaps 
were filled with T4 DNA polymerase before ligating, 
yielding plasmid pPB2395 (orfA- dsbA::TnphoA). 
Nucleotide sequence analysis indicated that the orfA 
reading frame was actually destroyed (data not shown). 
Bacteria of strain SBS2227 were transformed with either 
pPB2354 or pPB2395 and the AP activity of the plasmid-
borne dsbA::TnphoA fusion was measured in each case. 
Surprisingly, it was reduced by 30% by the frameshift 
mutation in pPB2395. However, when the second com-
patible plasmid pPB2144 (orfA+) was introduced into 
SBS2227(pPB2395), the presence of orfA+ in trans did not 
restore the original AP activity (Fig. 8). Consequently, the 
frameshift mutation very likely interferes with transcription 
and the orfA gene product itself is not necessary for 
dsbA expression.

**DISCUSSION**

The availability of MudII clones carrying the DNA 
region around dsbA led us to investigate in greater detail 
the characteristics of dsbA transcription. The analysis of 
the nucleotide sequence of the dsbA region reported here 
confirms, with some modifications, that the region lying 
upstream from dsbA corresponds to an open reading
frame, orfA, reported as o328 by Plunkett et al. (1993). However, we have described a transcriptional organization of the dsbA gene which is different from that proposed by Plunkett and coworkers. Our findings indicate that dsbA can be transcribed from an internal promoter located in the distal portion of orfA (o328). This promoter, P1, shows 66% sequence identity with the σ^70-dependent promoter consensus sequence. It can also be transcribed from another promoter, P2, located upstream from orfA. The P2 promoter, whose –10 box (TATccT) has 66% identity with the σ^70-dependent –10 box consensus sequence, does not show any typical and correctly spaced –35 box. However, measures of alkaline phosphatase activity in cultures of strains harbouring a plasmid with a dsbA::TnphoA fusion under the control of only one promoter (P1) or both promoters (P1 and P2) indicated that each promoter participates approximately equally to the transcription of the dsbA::TnphoA gene fusion. This means that the initiation of transcription at the –35 boxless promoter P2 is as efficient as that originating from the complete σ^70-dependent promoter P1. One noteworthy feature of the P2 promoter is the presence of an additional TGn motif just before its –10 box. It is known that such a motif can be sufficient to allow a strong initiation in vitro and in vivo from promoters lacking the –35 box but showing a ‘major’ –10 sequence. However non-major –10 boxes with imperfect nucleotides in the fourth and/or fifth position of the typical σ^70 consensus sequence (TATaaT) can also be functional (Kumar et al., 1993; Belyaeva et al., 1993). Raibaud & Schwartz (1984) also reported that positively controlled promoters generally contain little sequence similarity in the –35 region. However, the comparison of several reported consensus sequences corresponding to the binding sites of various transcription regulators (cAMP-CRP, Ebright, 1982; OmpR, Igarashi et al., 1991; TyrR, Yang et al., 1991; Lrp, Wang & Calvo, 1993; IHF, Goodrich et al., 1990) with the DNA sequence upstream from orfA did not show significant similarity. Studies on the regulation of the dsbA gene using chromosomal dsbA::TnlaZ fusion are now under investigation.

We have shown that orfA is actually a gene whose product has been identified as a 39 kDa protein, probably localized to the cytoplasm. The level of expression of this protein, which is independent of the orfA upstream region cloned, remained very low though the orfA P2 promoter was as active as the dsbA P1 promoter. Even when the orfA gene was placed under the control of the induced P_tac promoter, the production of the 39 kDa orfA gene product was not elevated (P. Belin & P. L. Boquet, unpublished result). This result suggests that the low expression of orfA does not result from a weak promoter activity but rather from a post-transcriptional event. Its very poor SD sequence is probably the cause of this low yield of protein synthesis.

The role of orfA was investigated by destruction of the chromosomal copy of orfA with an Ω fragment. This knock-out had no effect on growth and cell viability. The formation of disulfide bonds in the periplasmic space was not affected, even when substrate proteins were over-produced. The orfA gene product is thus not required for disulfide bond formation. Curiously, when the translation of orfA was aborted by introducing a frameshift mutation in the gene, the expression of a downstream dsbA::TnphoA fusion was reduced by about one-third. However, the presence in trans of several copies of the wild-type orfA gene did not restore the wild-type AP activity of the fusion. This result could be explained by a phenomenon of transcriptional polarity (Adhya & Gottesman, 1978) in which the absence of orfA translation could lead to the premature release of RNA polymerase from orfA. The physiological role of the orfA gene product remains to be elucidated.

Dsb-mediated disulfide bond formation appeared to be very efficient and allowed the overproduction of several cysteine-containing prokaryotic and eukaryotic proteins in the periplasmic space (AppA, Agp and PhoA, Belin et al., 1994; urokinkase-type plasminogen activator and tissue-type plasminogen activator, Bardwell et al., 1993). We have shown that reducing the amount of DsbA in the periplasmic space by limiting its transcription at promoter P1 on the single chromosomal gene does not change the efficiency of disulfide bond formation in the overproduced AppA, Agp and PhoA proteins. Thus, in a wild-type strain, DsbA is in excess for normal cell functions. The reduced state of DsbA is more stable than the oxidized one (Zapun et al., 1993; Wunderlich et al., 1993). One limiting factor of disulfide bond formation could be the kinetics of reoxidation of the DsbA protein after its contact with the membrane-bound DsbB protein and not the probability of DsbA–DsbB contact formation. Since in a wild-type strain more than half the DsbA is found in a reduced state in vivo (Bardwell et al., 1993), it is possible that reducing the total amount of DsbA by about the same factor mostly affects the pool of the reduced form with little change in that of the oxidized one. Experiments aimed to reduce the DsbA pool down to a critical threshold are now in progress.

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Dual promoter transcription of dsbA


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