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

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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;

Abbreviations: AP, alkaline phosphatase; SD, Shine–Dalgarno; Amp, ampicillin; Cm, chloramphenicol; Spc, spectinomycin; Str, streptomycin; Tet, tetracycline.

The EMBL accession number for the sequence data reported in this paper is X80762.

Table 1. Characteristics of strains and plasmids

Strain or plasmid	Description of the genotype	Source
<i>E. coli</i>		
CC118	F ⁻ Δ (<i>ara leu</i>)7697 Δ <i>lacX74</i> Δ <i>pboA20</i> <i>galE galK thi rpsE rpoB</i> <i>argE</i> (Am) <i>recA1</i>	C. Manoil (University of Washington, Seattle)
CSR603	F ⁻ <i>argE3 aro-14 galK2 leuB manI pbr-1 proA2 recA1 rpsL31 supE44</i> <i>thi thr-1 uvrA6 xyl-5</i>	E. Dassa (Pasteur Institute, Paris)
K10	HfrC <i>relA tonA22 pit-10</i>	B. Bachmann (Yale University, New Haven)
SBS2227	Same as K10, but <i>orfA::</i> Ω	This study
SBS2171	HfrC <i>relA pit-10 tonA22 thi polA12</i> (ts) Tn10-64 (Tn10 90% cotransducible with <i>polA</i>)	This study
SBS2280	Same as K10, but <i>dsbB::</i> Tn10	This laboratory
Plasmids		
pHSG575	Cm ^r	D. Touati (University Paris VII)
pHP45 Ω	Amp ^r Str ^r Spc ^r	J. P. Bohin (University Paris Sud)
pBR322	Amp ^r Tet ^r	J. Beckwith (Harvard Medical School, Boston)
pPB1132	Amp ^r <i>appB.A</i>	This laboratory
pEP1376	Amp ^r <i>agp</i>	This laboratory
pPB2142	Cm ^r <i>dsbA</i> (<i>HindIII</i> – <i>HindIII</i> insert into the <i>HindIII</i> site of pHSG575)	See Fig. 1
pPB2144	Cm ^r <i>mob orfA</i> (<i>EcoRI</i> – <i>PstI</i> insert into the <i>EcoRI</i> – <i>PstI</i> sites of pHSG575)	See Fig. 1
pPB2181	Amp ^r <i>dsbA71</i> (Oc)	This laboratory
pPB2196	Amp ^r <i>orfA dsbA</i> (<i>KpnI</i> *– <i>Clal</i> insert into the <i>HindIII</i> *– <i>Clal</i> sites of pBR322)	See Fig. 1
pPB2212	Amp ^r Tet ^r <i>dsbA</i> (<i>HindIII</i> – <i>SspI</i> insert into the <i>HindIII</i> – <i>NruI</i> sites of pBR322)	See Fig. 1
pPB2216	Amp ^r <i>orfA dsbA</i> (<i>KpnI</i> *– <i>SspI</i> insert into the <i>HindIII</i> *– <i>NruI</i> sites of pBR322)	See Fig. 1
pPB2217	Amp ^r Str ^r Spc ^r <i>orfA::</i> Ω <i>dsbA</i> (<i>Bam</i> HI Ω fragment inserted into the <i>Bam</i> HI site of <i>orfA</i> on pPB2216)	This study
pPB2234	Amp ^r <i>orfA dsbA</i> (<i>SmaI</i> – <i>SspI</i> insert into the <i>EcoRI</i> *– <i>NruI</i> sites of pBR322)	See Fig. 1
pPB2317	Amp ^r <i>dsbA::</i> Tn <i>pboA</i>	This laboratory
pPB2352	Amp ^r <i>orfA dsbA</i> (<i>NsiI</i> *– <i>SspI</i> fragment into the <i>EcoRI</i> *– <i>NruI</i> sites of pBR322)	This study
pPB2353	Amp ^r <i>orfA dsbA::</i> Tn <i>pboA</i> (<i>HpaI</i> fragment from pPB2317 into the <i>HpaI</i> sites of pPB2352)	See Fig. 5
pPB2354	Amp ^r <i>orfA dsbA::</i> Tn <i>pboA</i> (a 4.8 kb Δ <i>XhoI</i> deletion on plasmid pPB2353)	See Fig. 5
pPB2355	Amp ^r Str ^r Spc ^r <i>orfA dsbA::</i> Tn <i>pboA</i> (<i>SmaI</i> Ω fragment inserted into the <i>XhoI</i> * site of pPB2354)	See Fig. 5
pPB2358	Amp ^r Str ^r Spc ^r <i>orfA::</i> Ω <i>dsbA::</i> Tn <i>pboA</i> (<i>Bam</i> HI Ω fragment inserted into the <i>Bam</i> HI site of <i>orfA</i> on pPB2354)	See Fig. 5
pPB2359	Amp ^r Str ^r Spc ^r <i>orfA dsbA::</i> Tn <i>pboA</i> (<i>SmaI</i> Ω fragment inserted into the <i>SmaI</i> site of pPB2354)	See Fig. 5
pPB2360	Amp ^r Δ (P2) <i>orfA dsbA::</i> Tn <i>pboA</i> (a 113 nucleotide <i>SmaI</i> – <i>EcoRV</i> deletion on pPB2354)	This study
pPB2366	Amp ^r <i>dsbA</i> (a 0.9 kb Δ <i>HindIII</i> deletion on pPB2234)	See Fig. 1
pPB2394	Amp ^r Δ (P2- <i>orfA</i>) <i>dsbA::</i> Tn <i>pboA</i> (a 107 nucleotide <i>EcoRV</i> – <i>Bam</i> HI* deletion on pPB2354)	This study
pPB2395	Amp ^r <i>orfA' dsbA::</i> Tn <i>pboA</i> (<i>Bam</i> HI digestion, fill-in and ligation on pPB2354)	This study

* Sites that have been filled with T4 DNA polymerase.

Fig. 1. Restriction map of the *dsbA* region, deduced from the DNA sequence. Dashed lines correspond to unsequenced DNA. The solid arrows indicate the positions and orientations of the open reading frames; o89 has been described by Plunkett *et al.* (1993). Plasmid designations and DNA regions cloned are indicated. The Ω fragment is shown as a stippled box. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; N, *Nsi*I; P, *Pst*I; S, *Sma*I; Sn, *Sna*BI; Sp, *Ssp*I.

Dailey & Berg, 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_{vir} according to Miller (1972). Cells were grown in TYE medium (bacto tryptone, 10 g l⁻¹; yeast extract, 5 g l⁻¹; and NaCl, 8 g l⁻¹, pH 7.5) as a rich medium. Ampicillin (Amp) was added to a final concentration of 200 µg ml⁻¹ and chloramphenicol (Cm) at 10 µg ml⁻¹, unless otherwise stated. Streptomycin (Str), spectinomycin (Spc) and tetracycline (Tet) were added as indicated in the text. The induction of the P_{tac} 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 pHP45 Ω 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' GATTCAATAAAGTCTGACCGT 3' which is complementary to nucleotide position 272–292 of *orfA* (o328), and oligonucleotide 5' CGCGGCGTAATTAAAGAGTAAAA 3', which corresponds to a nucleotide sequence lying just before the *Hind*III 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 (Marck, 1988). Homologies were searched for in the NBRF databases (release number 38) by using the BISANCE facilities (Dessen *et al.*, 1990).

Maxicell analysis. Plasmids pBR322, pPB2196, pPB2234, pPB2181 and pPB2366 were introduced into strain CSR603 by transformation. Maxicells were prepared from transformants according to Sancar *et al.* (1979), and radioactive proteins were analysed by SDS-PAGE (Laemmli, 1970) and autoradiography.

RNA preparation and primer extension. RNA was isolated from strain CC118 carrying plasmid pPB2196 according to Aiba *et al.* (1981) with some modifications. The bacterial pellet from 2 ml of culture was resuspended in 200 µl 0.02 M sodium acetate (pH 5.5), 0.5% SDS, 0.001 M EDTA and 0.1% diethyl pyrocarbonate, then 400 µl of hot phenol equilibrated with 0.02 M sodium acetate (pH 5.5) was added and the bacterial suspension was incubated for 5 min at 60 °C after briefly vortexing. After centrifugation, the aqueous layer was decanted and re-extracted twice with hot phenol. RNAs were precipitated with 0.1 vols NaCl (5 M) and 3 vols ethanol, and the solution was left for 1 h at –70 °C. After centrifugation, the RNA pellet

cgatatcgctcttttattgtgggattgacctgctaactgtgtctgtctcaagagtaaggagcatcactatgaaatgtaa	755
acgtctgaatgaagttattgaactcctccagccagcctggcaaaaagagccagaccttaacctgctgcaatttttgcaga	835
aactggcgaaagagtcaggttttgacggcgaaactggcgatttgacggatgacattctgatctatcacctgaaaatgcgt	915
gattccgcgaaagatgcggtgatcccggtttgcagaaagattatgaagaagacttcaaaacggcgctgttacgcgctcg	995
cggcgtaattaagagtaaaagcttgtaagcgcgccaccaaatacatcgtaaatgatatccttcgtcattcgtaatgt	1075
SD tttccggatgatggg ATG AAT AAC AGC GCT TTT ACT TTC CAG ACA CTA CAC CCG GAT ACC ATC	1138
M N N S A F T F Q T L H P D T I	
ATG GAC GCT CTG TTT GAG CAT GGG ATC CGC GTG GAT TCC GGT CTT ACC CCG CTT AAC AGC	1198
M D A L F E H G I R V D S G L T P L N S	
TAT GAA AAC CGT GTC TAT CAA TTT CAG GAC GAA GAT CGT CGA CGT TTT GTC GTC AAA TTT	1258
Y E N R V Y Q F Q D E D R R R F V V K F	
TAT CGC CCT GAA CGT TGG ACA GCC GAT CAA ATC CTC GAA GAA CAT CAA TTT GCG TTG CAG	1318
Y R P E R W T A D Q I L E E H Q F A L Q	
CTG GTA AAT GAT GAA GTT CCG GTC GCA GCA CCT GTG GCC TTT AAC GGT CAG ACT TTA TTG	1378
L V N D E V P V A A P V A F N G Q T L L	
AAT CAT CAG GGA TTT TAT TTC GCT GTT TTT CCA AGC GTC GGT GGT CGC CAG TTC GAA GCT	1438
N H Q G F Y F A V F P S V G G R Q F E A	
GAT AAT ATC GAT CAG ATG GAA GCG GTT GGG CGT TAT TTA GGG CGT ATG CAC CAG ACG GGG	1498
D N I D Q M E A V G R Y L G R M H Q T G	
CGC AAA CAG CTT TTT ATC CAT CGC CCG ACC ATC GGT TTG AAC GAA TAT CTC ATT GAG CCA	1558
R K Q L F I H R P T I G L N E Y L I E P	
CGC AAG CTG TTT GAG GAC GCT ACA CTG ATA CCT TCC GGG TTG AAA GCG GCA TTC CTG AAA	1618
R K L F E D A T L I P S G L K A A F L K	
GCG ACA GAT GAG CTG ATT GCC GCC GTT ACA GCA CAC TGG CGG GAA GAT TTC ACC GTT CTG	1678
A T D E L I A A V T A H W R E D F T V L	
CGG CTA CAT GGA GAC TGC CAC GCC GGG AAT ATT CTC TGG CGC GAT GGT CCA ATG TTT GTT	1738
R L H G D C H A G N I L W R D G P M F V	
GAT CTG GAT GAT GCA CGT AAT GGT CCA GCC GTT CAG GAT TTG TGG ATG TTG CTC AAT GGC	1798
D L D D A R N G P A V Q D L W M L L N G	
GAT AAA GCC GAG CAG CGG ATG CAA CTG GAA ACT ATT ATT GAA GCT TAT GAA GAA TTT AGC	1858
D K A E Q R M Q L E T I I E A Y E E F S	
GAG TTC GAC ACC GCT GAA ATC GGA CTG ATT GAA CCT TTA CGC GCC ATG CGT TTG GTT TAT	1918
E F D T A E I G L I E P L R A M R L V Y	
TAT CTT GCC TGG CTA ATG CGG CGT TGG GCT GAT CCC GCG TTC CCG AAA AAT TTC CCG TGG	1978
Y L A W L M R R W A D P A F P K N F P W	
TTA ACC GGG GAA GAT TAC TGG CTG CGA CAG ACG GCG ACT TTT ATA GAA CAG GCA AAA GTT	2038
L T G E D Y W L R Q T A T F I E Q A K V	
-10 CTA CAA GAA CCC CCT TTG CAA TTA ACA CCT ATG TAT taatcgagagagtagatc ATG AAA AAG	2102
L Q E P P L Q L T P M Y M K K	
ATT TGG CTG GCG CTG GCT GGT TTA GTT	2129
I W L A L A G L V	

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' CTTCAAACTGATAGCAGTGC GGGC 3', which is complementary to position 146–169 of the *dsbA* gene, and 5' GGATCCCATGCTCAAACAGAGCGTCCATGAT 3', which is complementary to position 46–76 of *orfA*, were used. The oligomers were 5' end-labelled by using polynucleotide kinase and [γ - ^{32}P]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 MudII5117 has been reported previously and the recombinant clones carried 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, already described (Fig. 1; Belin *et al.*, 1994).

Nucleotide sequence analysis was performed on both DNA strands and extended from a *Bam*HI site lying 2.1 kb upstream of the *dsbA* ATG initiation codon to a *Hind*III 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 named *orfB* and encoding a putative polypeptide of 476 amino acid residues. The difference in length with o490 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 *p*-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 0.270 (Sharp & Li, 1987). The nucleotide sequence of *orfA* was found to be identical to o328 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 4015 TGATAATCCT 4024, while our sequence reads TGATATCT: the supernumerary 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* (o490), 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 *Hind*III 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 *Bam*HI restriction sites are indicated.

Fig. 3. Identification of the product encoded by the *orfA* and *dsbA* genes. Autoradiogram of an SDS-polyacrylamide gel of lysates prepared from ^{35}S -labelled maxicells obtained from strain CSR603 harbouring plasmid pBR322 (lane 1), pPB2196 (*orfA*⁺ *dsbA*⁺, lane 2), pPB2181 as control [*dsbA*71 (Oc), lane 3], pPB2234 (*orfA*⁺, *dsbA*⁺, lane 4) or pPB2366 (Δ *orfA* *dsbA*⁺, lane 5). Plasmid structures are indicated in Fig. 1 (note that plasmid pPB2181 does not carry the *orfA* region).

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 *Tn*lacZ 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 *o89* or *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 *Hind*III–*Ssp*I fragment inserted between the *Hind*III and *Nru*I sites of pBR322 (Fig. 1). Inserting a 2 kb Ω interposon which contains a *Str*^r and *Spc*^r gene flanked by transcription and translation termination signals, and multiple cloning sites (Prentki & Krisch, 1984) into the *Hind*III 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

Fig. 4. Primer extension analysis of the *dsbA* (a) and *orfA* (b) transcripts. Primers complementary to nucleotides 146–169 of *dsbA* and to nucleotides 46–76 of *orfA* were annealed to different amounts of total *E. coli* mRNA extracted from bacteria of strain CC118 harbouring plasmid pPB2196, and extended with moloney murine leukemia virus reverse transcriptase. The sequence ladders obtained using the same primers were run as size standards (lanes T, G, C and A). Arrows indicate initiation sites (see Results). (a) Total RNA (0.5 µg) was used for *dsbA* and lanes 1A and 1B correspond to 20 h and 3 h exposure time, respectively. (b) For *orfA*, 0.5 µg (lane 1), 5 µg (lane 2) and 25 µg (lane 3) total RNA was used.

promoter. Consequently the *Hind*III–*Ssp*I 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

Fig. 5. Linear representation of the constructed plasmids carrying a *dsbA::phoA* fusion. The thick hatched arrow indicates the *dsbA* gene, and the thin solid arrow the *'phoA* gene of *TnphoA*. The dash-dot arrow is the *orfA* gene. The thick solid bars are the IS regions of the *TnphoA* transposon. The Ω fragment is indicated as a box. Thin dashed lines correspond to vector sequences. The restriction sites in parentheses are those which have been lost in the construction of the plasmids.

extracted from cells of strain CC118 harbouring pPB2196. mRNA (0.5 µg) was first hybridized with a ^{32}P -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 σ^{70} -dependent consensus box. The predicted –35 box (TTTATA) is 16 nucleotides upstream of the –10 box and also shows 66% identity with the –35 σ^{70} -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 TTGTAA located 29 nucleotides upstream from the –10 box, a distance which is not typical for a σ^{70} -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*

Fig. 6. Differential rate of alkaline phosphatase synthesis originating from plasmids carrying the *dsbA::phoA* fusion. Bacteria of strain CC118 carrying plasmids pPB2354 (■), pPB2355 (□), pPB2358 (●), pPB2359 (▲), pPB2360 (○) and pPB2394 (△) were grown in TYE medium at 37 °C in the presence of Amp (200 µg ml⁻¹) and alkaline phosphatase activity was measured as described in Methods. Each point represents the mean of five results made on independently collected samples. The SEM was calculated and found to be < 2% for each measure (not represented). In each case, the slope (*s*) and the coefficient of correlation (*r*²) are indicated.

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 *dsbA::Tnp_{phoA}* downstream fusion. One such fusion obtained by *Tnp_{phoA}* insertion between nucleotides 79 and 80 of the *dsbA* gene has already been described and is carried on plasmid pPB2317 (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 the periplasmic space. The *dsbA::Tnp_{phoA}* gene fusion was isolated from plasmid pPB2317 on a 7.7 kb *HpaI* fragment and was inserted between the *HpaI* sites of plasmid pPB2352 (see Fig. 1), yielding plasmid pPB2353. This plasmid which carries the fusion, the whole *orfA* gene and part of the *mob* sequence was shortened by *XhoI* deletion to remove the transposase activity and some redundant restriction sites, yielding pPB2354 (Fig. 5). The Ω interposon was inserted into pPB2354 at different positions: at the *XhoI* site downstream from the fusion (pPB2355), at the *BamHI* site located in the *orfA* gene (pPB2358) and at the *SmaI* site lying upstream from the *orfA* promoter (pPB2359). Each of these plasmids was introduced into bacteria of strain CC118 (*dsbA*⁺ *ΔphoA*).

Transformants were grown on TYE at 37 °C and AP activity was measured. Insertion of the Ω fragment outside of the *orfA-dsbA* region did not change AP activity relative to that observed with the original plasmid pPB2354. In contrast, interruption of transcription within the *orfA* gene in pPB2358 reduced AP activity by about 50 % at all stages of growth (compare the slopes of the lines on Fig. 6).

Two deletions were made around the *orfA* promoter on plasmid pPB2354 and the activity of AP in cultures of strain CC118 containing each plasmid was measured. The first deletion extends from the *SmaI* 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 Ω 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 (*polA*_{ts}) 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 42 °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^r Spc^r (10 µg ml⁻¹ each). A Tet^r Str^r Spc^r and *polA*⁺ transductant (SBS2227) was selected. Further transduction experiments made with this strain as donor showed that the Str^r Spc^r 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

Fig. 7. Effect of the insertion of the Ω interposon in the *orfA* chromosomal gene on the production of the periplasmic DsbA. (a) Silver-stained SDS-polyacrylamide gel (13% acrylamide) run with shock fluids from bacteria of strains K10 (wild-type, lane 1), SBS2096 as control [*dsbA*71 (Oc), lane 2], SBS2227 (*orfA*:: Ω *dsbA*⁺, lane 3) and SBS2280 (*dsbB*::Tn10, lane 4) as control. (b) Similar staining of runs with shock fluids of K10(pHSG575) as control (lane 1), SBS2227(pHSG575) as control (lane 2) and SBS2227(pPB2144) (lane 3). pHSG575 is only the vector and pPB2144 carries the *orfA*⁺ gene and a truncated *dsbA*' fragment encoding an inactive polypeptide (Belin *et al.*, 1994).

shown). This fragment, when religated into the *Bam*HI 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 phosphatases was examined. The activities of pH 2.5 acid phosphatase (AppA), glucose-1-phosphatase (Agp) and alkaline phosphatase (PhoA) were the same in SBS2227 and K10 (data not shown). Moreover, when bacteria of both strains were transformed with plasmids overproducing the same phosphatases, namely pPB1132 for AppA (Boquet *et al.*, 1987), pEP1376 for Agp (Pradel & Boquet, 1988) and pLIP5.0 for PhoA (*phoA* under the control of the *P*_{tac} 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 experiments were performed. Firstly, bacteria of strain SBS2227 were transformed with plasmid pPB2144 which carries the entire *orfA* gene cloned into the vector pHSG575. 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*::Tn*phoA*) was mutated only by frameshifting to try to avoid a block of its transcription from the P2 promoter. The plasmid was cut with *Bam*HI and the gaps were filled with T4 DNA polymerase before ligating, yielding plasmid pPB2395 (*orfA*⁻ *dsbA*::Tn*phoA*). 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*::Tn*phoA* fusion was measured in each case. Surprisingly, it was reduced by 30% by the frameshift mutation in pPB2395. However, when the second com-

Fig. 8. Expression of alkaline phosphatase originating from the plasmid-borne *dsbA*::*phoA* fusion in a strain carrying an *orfA*:: Ω insertion. Bacteria of strain SBS2227 carrying plasmids pPB2354 (▲), pPB2395 (■), and pPB2395 and pPB2144 (●) were grown in TYE medium at 37 °C with Amp (200 µg ml⁻¹) and alkaline phosphatase activity was measured and expressed as in Fig. 6.

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::TnlacZ* 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 cystine-containing prokaryotic and eukaryotic proteins in the periplasmic space (AppA, Agp and PhoA, Belin *et al.*, 1994; urokinase-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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