JOURNAL OF BACTERIOLOGY, Jan. 1995, p. 462–464 Vol. 177, No. 2
0021-9193/95/$04.00+0

A Mutation in Either *dsbA* or *dsbB*, a Gene Encoding a Component of a Periplasmic Disulfide Bond-Catalyzing System, Is Required for High-Level Expression of the *Bacteroides fragilis* Metallo-β-Lactamase, CcrA, in *Escherichia coli*

LEFA E. ALKSNE, DAVID KEENEY, AND BETH A. RASMUSSEN*

Medical Research Division, Department of Molecular Biology, American Cyanamid Company, Pearl River, New York 10965

Received 22 June 1994/Accepted 9 November 1994

The metallo-β-lactamase gene, *ccrA*, from *Bacteroides fragilis* is functionally expressed in *Escherichia coli* only in the presence of a genomic mutation in *iarA* or *iarB* (increased ampicillin resistance), identified in this study as *dsbA* or *dsbB*, respectively. DsbA and DsbB are components of a periplasmic protein disulfide bond-catalyzing system. Data indicated that DsbA interacted with CcrA, creating aberrant disulfide bond linkages that render CcrA proteolytically unstable. Mutations in *dsbA* or *dsbB* permissive for CcrA expression eliminated or greatly reduced DsbA activity, allowing CcrA to assume a disulfide bond-free and proteolytically stable conformation.

Expression of foreign proteins in *Escherichia coli* is not always successful. Multiple steps are required for the functional expression of a protein, including transcription, translation, translocation and processing, and correct secondary and tertiary folding. Inefficiency at any one of these steps may severely impair functional expression. Expression of the metallo-β-lactamase, CcrA, from *Bacteroides fragilis* is inefficient in a wild-type *E. coli* strain (18, 19). However, the level of functional expression increases greatly when the cells harbor a chromosomal mutation mapping to one of two *E. coli* chromosomal locations. These mutations were designated *iar* mutations, for increased ampicillin resistance (19), and fell into two distinct classes on the basis of the chromosomal location of the mutant alleles. Class I and class II mutations have been designated to lie within the *iarA* locus and the *iarB* locus, identified here as *dsbA* and *dsbB*, respectively (1, 4, 5, 9, 15, 17) (see below). The mechanism by which a mutation in *dsbA* or *dsbB*, a gene encoding a component of a disulfide bond-catalyzing system (3), yielded increased β-lactamase activity is the focus of this study.

Identification of block in expression of CcrA. The *dsb* mutations studied here bestow to cells harboring *ccrA* the phenotype of increased ampicillin resistance. *E. coli dsbA* or *dsbB* strains were 8- to 16-fold less susceptible to ampicillin than wild-type strains as determined by measurement of the MIC of ampicillin (19). It was demonstrated previously that this difference in ampicillin susceptibility is the result of increased β-lactamase activity in the *dsb* mutant strains (19).

The block in CcrA expression was determined to be a post-translational event. Although replacement of the *ccrA* promoter with the *lacUV5* promoter (12) resulted in an increase in the basal level of β-lactamase activity, the presence of a *dsbA* or *dsbB* mutation still gave an 8- to 16-fold increase in the level of ampicillin susceptibility (data not shown). Similarly, replacement of the *ccrA* promoter and the signal sequence with the *lacUV5* promoter and signal sequence-coding region from *malE*, encoding the periplasmic maltose-binding protein (8, 21), also did not relieve the *dsb* effect on *ccrA* expression (data not shown). Therefore, the *dsb* mutations functioned at a step after transcription, translation, and the early steps in signal sequence recognition and entrance into the export pathway.

Identification of increased ampicillin resistance mutations as mutations in *dsbA* and *dsbB*. The class I and class II mutations were mapped by P1 cotransduction (14, 25) to 25.5 and 87 min on the *E. coli* K-12 chromosome, respectively (data not shown). When the locations were initially determined, no genes had been identified within either of the two regions (2) whose function would suggest a possible mechanism for the observed increase in β-lactamase activity. Therefore, the class I and class II loci were cloned.

The wild-type class II locus, *iarB*, was cloned by screening for complementation of the β-lactamase-positive phenotype caused by the *iarB1* mutation. From total chromosomal DNA cloned into pACYC184 (7, 23), a 9-kb *Bam*HI fragment which, when transformed into a CcrA-expressing cell line, bestowed a β-lactamase-negative phenotype was identified. This pACYC184 (7, 23) plasmid derivative was designated pCLL*iarB*. The class I locus, *iarA*, was cloned following inactivation of the gene by Tn*10* insertion (10, 24), which resulted in a β-lactamase-positive phenotype. The locus was then cloned as two *Eco*RI fragments into pUC119 (26), by using radioactive probes to Tn*10* to identify it within a genomic bank (data not shown). DNA sequence analysis of the two clones and homology searches were performed by using the computer program DNAStar (DNASTAR, Inc., Madison, Wis.). The deduced amino acid sequences of the two genes were compared with sequences in the Swiss Protein Data Bank (release 80) by using the Pearson and Lipman algorithm (16). Both the DNA and amino acid sequence analyses confirmed that *iarA* was *dsbA* and that *iarB* was *dsbB* (1, 4, 5, 9, 15, 17). Analysis of several *dsbA* and *dsbB* mutant alleles (data not shown) indicated that these mutations were null mutations in either *dsbA* or *dsbB*.

The CcrA protein has an increased half-life in a *dsb* mutant strain. The level of translation and the half-life of CcrA were examined in *dsbA1*, *dsbB1*, and wild-type derivatives of MC4100 (6). Cell cultures grown to mid-log phase in M63

* Corresponding author. Mailing address: American Cyanamid Company, Building 205, Room 214, Pearl River, NY 10965. Phone: (914) 732-4569. Fax: (914) 732-2480.

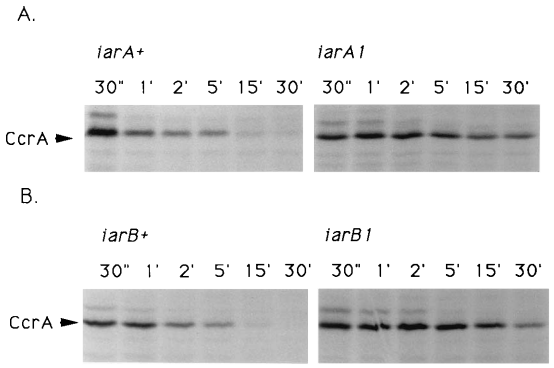

FIG. 1. Pulse-chase analysis of CcrA half-life in MC4100 (6) wild-type and *iar* (*dsb*) mutant strains. *iarA*$^+$ and *iarA1* (*dsbA1*) (A) and *iarB*$^+$ and *iarB1* (*dsbB1*) (B) strains expressing CcrA from the *lacUV5* promoter were pulse labeled for 1 min with [^{35}S]methionine and chased with excess cold methionine beginning at time zero. At 30 s, and 1, 2, 5, 15, and 30 min postchase, the cells were arrested, and CcrA was immune precipitated and analyzed by SDS-PAGE and autoradiography.

glucose were pulse labeled (1 min) with [^{35}S]methionine (22 μCi/ml, >1,000 mCi/mmol; New England Nuclear, Boston, Mass.). The incorporation of radiolabel was terminated by chasing with cold, unlabeled methionine (21). At various times postchase, an aliquot of the culture was removed and precipitated with cold trichloroacetic acid (final concentration, 5%). The precipitate was harvested, washed with acetone, and solubilized as described previously (21). Radiolabeled CcrA was analyzed by immunoprecipitation with rabbit polyclonal antiserum prepared against purified protein (22), followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11) and autoradiography. The amounts of CcrA produced in a *dsb*$^+$ strain and a *dsbA1* or *dsbB1* strain were comparable (Fig. 1, compare lanes labeled 30″); however, the half-lives of the CcrA proteins were significantly different. When CcrA was expressed in a *dsb*$^+$ strain, the average half-life for CcrA was less than or equal to 5 min (Fig. 1). Densitometry analysis using a GS300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, Calif.) indicated that there were two populations of CcrA present in the *dsb*$^+$ strains; the majority had a very short half-life, while a minor population had a long half-life similar to that of CcrA produced in a *dsb* mutant strain. When CcrA was expressed in either a *dsbA1* or *dsbB1* strain, only one CcrA population was observed. This population had a half-life of 15 to 30 min (Fig. 1). Thus, the block in β-lactamase expression was indeed a posttranslational event, with rapid proteolysis being a possible contributing factor to the low levels of enzymatic activity.

Of additional interest is the observation that there is more stable CcrA precursor present in the *dsb* mutant strains than in the wild-type strains. This suggests that the *dsb* mutations may directly or indirectly affect signal sequence processing. It is unclear whether this inhibition is CcrA specific or of a more general nature.

Determination of reduced state of CcrA. CcrA contains three cysteine residues, one of which is proposed to participate in Zn^{2+} binding (18). Since disruption of *dsbA* resulted in a β-lactamase-positive phenotype, it was hypothesized that DsbA was interacting with CcrA, creating aberrant disulfide bonds. To determine if this was a feasible hypothesis, the reduced state of the three cysteine residues in enzymatically active recombinant CcrA was determined. Reaction of native or denatured CcrA with 5,5′-dithiobis(2 nitrobenzoic acid) (DTNB) (13, 29) indicated the presence of three free cysteine residues (data not shown). This was confirmed by reaction of CcrA with iodoacetamide followed by molecular weight determination (data not shown). Thus, in enzymatically active CcrA, none of the cysteine residues were disulfide bonded.

Western analysis of CcrA. Western (immunoblot) analysis of CcrA in whole-cell extracts prepared from *dsb*$^+$, *dsbA1*, and *dsbB1* derivatives of MC1061 (6) or MC4100 (6) was performed. Cell extracts were prepared from mid-logarithmic-phase cultures. The cells were harvested, resuspended in sample buffer with or without β-mercaptoethanol (11), and placed in a boiling water bath for 5 min to disrupt the cells. These extracts were subjected to SDS–11% PAGE (11). The proteins were transferred from the polyacrylamide gel to a nitrocellulose filter by using an LKB Multiphor II system. Western analysis was done with rabbit polyclonal anti-CcrA antiserum and the ECL Western blotting chemiluminescent detection system (Amersham Corporation, Arlington Heights, Ill). CcrA was expressed from either the *B. fragilis* IS*942* promoter (20) or the *lacUV5* promoter (12).

When the cell extracts were prepared and maintained under reducing conditions, two discrete CcrA protein bands, migrating in the gel at the positions of the mature and precursor (with a signal sequence) proteins, were observed. This was the case for CcrA from both wild-type and *dsb* mutant strains. There was, however, notably less CcrA in the extracts prepared from *dsb*$^+$ strains. This correlated with the shorter half-life of CcrA in a *dsb*$^+$ background. Although the level of CcrA expressed from the IS*942* promoter was lower than that expressed from the *lacUV5* promoter, the relative levels of CcrA expressed from each promoter were the same for all the *dsb* strains.

In contrast, the CcrA-banding patterns were strikingly different when CcrA was expressed from the *lacUV5* promoter in a wild-type or *dsb* mutant strain and the cell extracts were prepared under nonreducing conditions. Western analysis of extracts prepared from *dsb*$^+$ cells revealed only a very small amount of CcrA migrating at the expected position on the gel, less than that seen with the identical sample processed under reducing conditions. In addition, significantly more slowly migrating forms that reacted with the antibody appeared, as both discrete bands and smudges or streaks on the gel (Fig. 2, lanes 11 and 15). Similar more slowly migrating forms were not seen in the nonreduced extracts prepared from *dsbA1* or *dsbB1* strains. These more slowly migrating forms corresponded to CcrA proteins disulfide bonded by DsbA either to themselves or to another protein(s); the smudging or band trailing was probably the result of proteolysis of these aberrant forms of CcrA.

We have demonstrated that the block in functional expression of CcrA in *E. coli* is a posttranslational event, independent of any promoter recognition or translocation problems. Effective expression of CcrA required a chromosomal mutation at one of two loci, identified in this work to be *dsbA* and *dsbB*. The presence of functional DsbA and DsbB proteins resulted in the creation of aberrant disulfide bonds that greatly affected the half-life of CcrA and perhaps its enzymatic activity. This cannot be overcome by growing *E. coli* in a reduced environment; a *dsbA* mutation was still required for functional expression of CcrA when *E. coli* was grown anaerobically (data not shown).

It has been demonstrated that DsbA can facilitate the formation of correct disulfide bond linkages in foreign proteins both in vitro and in vivo (1, 5, 9, 27, 28). However, this is the first report of DsbA catalyzing the formation of aberrant disulfide bonds in a protein. As expression of CcrA in *E. coli* illustrates, the use of *E. coli dsb* mutant strains could be efficacious in the cloning and extracytoplasmic expression of for-

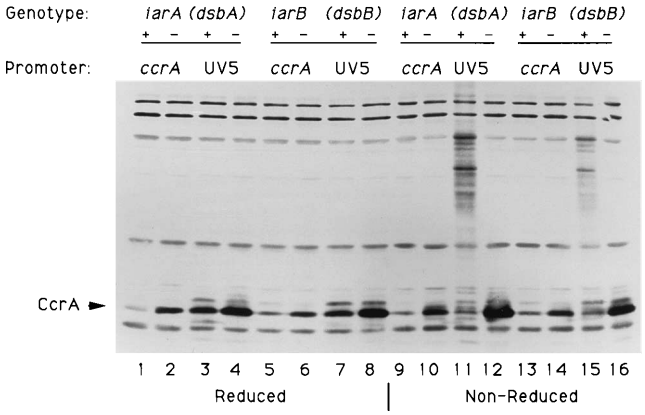

FIG. 2. Western analysis of CcrA under reducing and nonreducing conditions. Western analysis of whole-cell extracts prepared from MC1061 (6) wild-type, *iarA1* (*dsbA1*), and *iarB1* (*dsbB1*) strains was performed with CcrA expressed from either an IS*942* promoter (20) or the *lacUV5* promoter (12). CcrA was identified by using anti-CcrA polyclonal rabbit antibody (22) and visualized by chemiluminescence with X-ray film. Lanes 1 to 8 contain whole-cell extracts prepared under reducing conditions, while lanes 9 to 16 contain the same extracts prepared under nonreducing conditions. The position of mature CcrA protein is indicated by the arrowhead. An additional band migrating more slowly than mature CcrA and running at the position of precursor CcrA, with the signal sequence attached, is often seen in cell extracts prepared from cells producing higher levels of CcrA. A set of five strong and several weak background bands is always seen with all *E. coli* cellular extracts whether expressing CcrA or not (data not shown). These bands are consistent in intensity and are present in all 16 lanes of this figure.

eign proteins such as proteins from anaerobes or proteins that naturally reside in a reduced environment.

We thank Andrew Seddon for performing the iodoacetamide determination of the free cysteine content of CcrA, Margareta Tuckman for providing Tn*10* probes, and Steve Projan for critical reading of the manuscript.

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