Defect in export and synthesis of the periplasmic galactose receptor MglB in dnaK mutants of Escherichia coli, and decreased stability of the mglB mRNA

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The high-affinity galactose permease, which comprises the periplasmic galactose receptor MglB, the membrane translocator MglC and the membrane-associated ATPase MglA, displayed a reduced activity in a dnaK temperature-sensitive mutant of Escherichia coli. This reduced transport activity correlated with a reduction in the quantity of MglB. At 42 °C, an accumulation of pre-MglB in the dnaK temperature-sensitive mutant reflected a defect in MglB export. In addition, an accumulation of pre-MglB in secB, secA and secY mutants suggested that SecB and the Sec translocase are also involved in export of the periplasmic galactose receptor. At 30 °C, there was no accumulation of pre-MglB in the dnaK mutant, but there was still a decreased amount of MglB in the periplasm. The reduction in MglB expression was not the result of a decrease in its stability, nor was it the result of a general defect in translation or transcription, since the MglA protein (which is expressed from the same operon as MglB) was synthesized in normal amounts. Two mRNAs are implicated in the expression of the mgl genes, a polycistronic mglBAC mRNA, and a more stable and more abundant mglB mRNA, produced by 3'→5' degradation of the mglBAC mRNA (R. W. Hogg, C. Voelker & I. von Carlowitz, 1991, Mol Gen Genet 229, 453–459). The mglB mRNA is protected against exonucleases by a REP (Repetitive Extragenic Palindrome) sequence located at its 3' extremity, which is responsible for the higher expression of MglB compared to MglA and MglC. The decreased MglB expression in the dnaK mutant at 30 °C in the present work correlated with a reduced stability of the mglB mRNA, which may have resulted from a defective stabilization by the REP sequence, or from a defect in translation of the mglB gene.

**Keywords:** heat shock protein DnaK, protein export, mRNA stability, Escherichia coli dnaK mutants, periplasmic galactose receptor

**INTRODUCTION**

Molecular chaperones form a class of polypeptide-binding proteins that are implicated in protein folding, protein targeting to membranes, protein renaturation or degradation after stress, and control of protein–protein interactions (reviewed in Ellis & van der Vies, 1991; Georgopoulos et al., 1994; Hendrick & Hartl, 1993). The major classes of molecular chaperones of *Escherichia coli* comprise DnaK/hsp70 (and its assistants DnaJ and GrpE), GroEL/hsp60 (and its assistant GroES) and HtpG/hsp90. The heat shock protein DnaK/hsp70 (Georgopoulos, 1977; Bardwell & Craig, 1984; Gross et al., 1990; Bukau & Walker, 1989) plays a role in the maintenance of bacterial viability under stress, in addition to normal cellular functions. DnaK is a cytoplasmic protein, associated in part with the cytoplasmic membrane (Bukau et al., 1993; El Yaagoubi et al., 1994), which behaves as an ATP-dependent chaperone, facilitating the correct assembly or disassembly of certain oligomeric protein complexes, and participating in protein folding and renaturation and in the transmembrane targeting of certain proteins (Wild et al., 1992). DnaK has also been implicated in cell division (Bukau & Walker, 1989), murein synthesis (Wu et al., 1992), flagellar assembly (Shi...
Protein export across the *E. coli* cytoplasmic membrane involves principally the Sec transport apparatus, which is composed of the SecB chaperone, and of a translocase comprising the SecA ATPase and the membrane-embedded SecF/SecY complex (Economou & Wickner, 1994). Whereas SecB is considered to be the main chaperone for protein export, several pre-proteins are maintained in a translocation-competent state by the molecular chaperones DnaK/DnaJ (pre-PhoA) or GroEL/GroES (pre-ß-lactamase), and these molecular chaperones probably substitute for SecB under conditions in which SecB becomes limiting (Wild et al., 1992).

Furthermore, a signal recognition particle, similar to that found in eukaryotes, and which consists of at least one protein (Ffh) and one RNA molecule (4.5S RNA), functions as a chaperone specific for signal sequences in nascent pre-proteins and maintains their translocation-competent conformation (Luirink & Dobberstein, 1994).

The high-affinity galactose transport system of *E. coli* consists of the periplasmic galactose receptor MglB, an integral membrane protein MglC and the membrane-competent conformation (Luirink & Dobberstein, 1994). Protein export across the cytoplasmic membrane is transcribed into an unstable polycistronic mRNA. This is degraded in the cytoplasmic membrane by centrifugation at 10000 g for 10 min. The cell lysate was prepared by ultrasonic disruption of bacteria in 50 mM potassium phosphate, pH 6.8 (Branson Sonic Power ultrasonicator, 25 W, five times for 15 sec each), followed by centrifugation of the mixture at 40000 g for 15 min.

**Immunobots.** Antiseria against MglB and MglA were prepared in rabbits by immunization with 50 µg protein in Freund’s complete adjuvant. A booster immunization containing 50 µg protein in Freund’s incomplete adjuvant was administered after 3 weeks. Bleeding was performed 10 d after the injection. SDS-PAGE and immunobots were carried out according to Towbin et al. (1979). The intensity of protein bands was quantified with a Quick Scan densitometer (Helena Laboratories). MglB and MglA were purified as described previously (Richarme, 1983; Richarme et al., 1993).

**Radiolabelling, immunoprecipitation and electrophoresis.** Samples (1 ml) of cells growing exponentially at 30 °C in glycerol medium were pulse-labelled with 60 µCi ml⁻¹ [³⁵S]methionine (Amersham, 1000 Ci mmole⁻¹), chased with nonradioactive l-methionine (200 µg ml⁻¹) and precipitated with TCA as described by Ito et al. (1981). The precursor and mature forms of MglB were immunoprecipitated, resolved by electrophoresis on SDS-polyacrylamide gels [10 % (w/v) acrylamide], detected by autoradiography and quantified with an Ambis Scanner interfaced with an IBM computer.

**RNA techniques.** RNA for Northern blots was isolated by hot SDS/phenol extraction (Miller, 1972). Samples were separated on formaldehyde-agarose gels, transferred to nitrocellulose paper and hybridized to radiolabelled DNA probes in 50 % (v/v) formamide as described by Maniatis et al. (1982). The size of RNA species was estimated by comparison with rRNA and denatured DNA markers. The probe used was composed of a 22 residue synthetic oligonucleotide complementary to residues 396–419 of the mature mRNA (Hogg et al., 1991). It was labelled with [³²P]ATP by polynucleotide kinase as described by Maniatis et al. (1982). Rifampicin was used at 200 µg ml⁻¹, a high concentration currently used for the inhibition of transcription initiation in *E. coli* in vivo.

**METHODS**

**Bacterial strains.** *E. coli* K-12 strains C600 (lacB6 thi-1 thr-1 mps-44), WM1389 (C600 dnaK756 [ts]), MCA100 (F⁺ lacU169 araD139 thi-1 recA1 relA1), MM52 [MC4100 secA3 [ts]] (C600 secA51 [ts]), CK1953 (MC4100 secB1 [ts]), IQ86 (MC4100, Tn10) and IQ85 [IQ86 secY24 [ts]] were obtained from the laboratories of Drs J. Beckwith, K. Ito, C. Kumamoto, J. C. Walker and C. Georgopoulos. The strains were grown at 30 °C in glycerol minimal medium. The dnaK, secA and secY temperature-sensitive mutants were shifted to 42 °C for several hours (as indicated) before the experiments. The high-affinity galactose transport system was induced with 1 mM fucose.

**Transport measurements.** The bacteria were washed twice at 0 °C with minimal medium M63 (Miller, 1972) containing 0.4 % glycerol and resuspended in the same medium. The washed bacteria were incubated for 10 min at 22 °C before transport measurements. Galactose transport was assayed at 22 °C in a volume of 1 ml containing the equivalent of 50 µg of cell protein per ml of [³⁵S]galactose (250 µCi mmole⁻¹, Amersham) was added to the bacterial suspension to a concentration of 2 µM. A 200 µl sample of the assay mixture was removed from each flask at various times after the addition of the radioactive substrate and filtered through cellulose ester filters (Millipore HAWP, 0.45 µm pore size). The filters were washed three times with 1 ml of the transport medium and the radioactivity measured.

**RESULTS**

**Decreased galactose transport and decreased amount of the periplasmic galactose receptor in dnaK mutants**

The *dnaK*756 thermosensitive mutant is defective in galactose transport. When transport was measured after growth at the permissive temperature (30 °C), the transport activity was sevenfold lower in the mutant than in the parental strain (Fig. 1a). When transport was measured after growth at 30 °C, followed by a shift to 42 °C for 2 h, the transport activity was tenfold lower in the mutant (Fig. 1a). The defect in galactose transport correlated with a reduction in the amount of periplasmic galactose receptor in the *dnaK* mutant. The amounts of MglB in the periplasm of the *dnaK*756 mutant and of the parental strain C600 were measured by immunoblotting the periplasmic
fractons from both strains with anti-MglB antibodies. The amounts of MglB were fivefold lower in the dnaK mutant than in the parental strain at 30 °C (Fig. 1b) and sixfold lower at 42 °C (not shown). The correlation between reduced transport activity and a reduction in the amount of galactose receptor is consistent with the previous demonstration that the periplasmic receptor of a binding-protein-dependent transport system is reduced to less than 20% of its wild-type level (while the other transport components remain at the wild-type level (see below)), it becomes limiting for transport (Manson et al., 1985). Similar reductions in galactose transport and in galactose receptor expression were found in the null dnaK mutant GW 4813 (ΔdnaK52::Cm)
(Paek & Walker, 1987) (not shown), suggesting that these defects are common to several dnaK mutants.

**Defect in MglB export in the dnaK temperature-sensitive mutant at 42 °C**

The efficient export of the galactose receptor to the periplasm of *E. coli* has been recently shown to be dependent on the presence of the SecB chaperone (Powers & Randall, 1995). However, the following results suggest that dnaK mutations also affect the export of MglB. At 42 °C, under conditions in which the parental strain contains only mature galactose receptor (pulse-labeling for 15 sec, chase for 1 min), the dnaK temperature-sensitive mutant contains a significant amount of the galactose receptor as the precursor form (Fig. 2a). This secretion defect is not observed at 30 °C, at which temperature the MglB precursor does not accumulate either in the mutant or in the parental strain (Fig. 2a). It should be noted that at both 30 °C and 42 °C, the amount of MglB synthesized is reduced in the dnaK mutant (see below). The half-time for complete export of MglB to the periplasm at 42 °C was approximately 1 min in the dnaK mutant, compared to less than 10 sec for the parental strain C600 (Fig. 2b), while at 30 °C, the kinetics of MglB export were similar in the mutant and in the parental strain. Thus, dnaK756 appears to be defective in its secretion of the galactose receptor at the restrictive

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**Fig. 1.** (a) Decreased galactose transport in the dnaK756 mutant. Galactose transport was measured as described in Methods at 22 °C in the wild-type strain C600 (■, ■) and in the dnaK756 mutant (○, ●), grown at 30 °C (□, ○) or grown at 30 °C followed by a shift to 42 °C for 2 h (■, ○). (b) Decreased amount of the MglB protein in the dnaK756 mutant. Periplasmic fractions from strains C600 and dnaK756, grown at 30 °C, were prepared as described in Methods. The fractions were subjected to electrophoresis on a polyacrylamide gel and immunoblotted with anti-MglB antibodies. Columns 1 and 2 contain, respectively, 7 and 20 μg of the shock fluid from the parental strain C600. Columns 3 and 4 contain, respectively, 7 and 20 μg of the shock fluid from the dnaK756 mutant.

**Fig. 2.** (a) MglB precursor in the dnaK mutant at 42 °C. Cells growing exponentially in glycerol medium at 30 °C (experiment indicated 30 °C), or growing at 30 °C to OD 0.7 and then transferred to 42 °C for 1 h (experiment indicated 42 °C), were pulse-labelled for 15 s with [35S]methionine, chased with nonradioactive methionine for 30 s and precipitated with TCA. The galactose receptor was prepared from each sample by immunoprecipitation. (b) Kinetics of MglB export. Experiments were carried out as described in the legend to Fig. 2(a). Samples were pulse-labelled for 15 s and chased for the indicated times. The data are the average from two experiments. □, Strain C600 at 30 °C (□), or 42 °C (■), ○, □ dnaK756 mutant at 30 °C (□) or 42 °C (●). Since the radiolabel was methionine and three of the nine methionine residues are in the leader sequence, a factor of 1.5 was used to convert the radioactivity recovered in the mature form to the amount of precursor polypeptide from which it was derived.
temperature, but not at 30 °C. While most of the exported proteins of L. coli interact with the SecB chaperone (Wild et al., 1992; Kumamoto & Beckwith, 1983), some of them interact alternatively (MalF, LamB) or preferentially (alkaline phosphatase, PhoA) with DnaK (Wild et al., 1992). Since DnaK is involved in many cellular processes, we cannot completely exclude an indirect effect of the dnaK mutation on MglB export. However, the delay in the processing of pre-MglB to the mature species was already apparent after a 40 min shift of the thermosensitive mutant at 42 °C (not shown), before the cells stopped growing (growth was severely impaired after 90 min and stopped after 3 h). Furthermore, the SecB-dependent export of several proteins (OmpA, LamB) is not altered in dnaK mutants (Wild et al., 1992), suggesting that dnaK mutations do not affect the expression or the assembly of the Sec export apparatus.

Involvement of the Sec translocase in MglB export

The results shown in Fig. 3 indicate that, in addition to SecB and DnaK, the SecA and SecY components of the cellular export apparatus are involved in the export of MglB. The MglB precursor accumulates in the secB (IQ86), secA (MM52) and secY (Kumamoto & Beckwith, 1983; Ito et al., 1983; Oliver & Beckwith, 1981) mutants, in conditions where the parental strains do not accumulate significant amounts of the precursor (Fig. 3). This suggests that the role played by DnaK in MglB export could be that of a chaperone functioning in parallel with SecB (or perhaps before SecB), and that membrane translocation of MglB requires the Sec translocase.

Decreased MglB synthesis in the dnaK mutant at 30 °C and 42 °C

Further analysis of Fig. 2a shows that MglB expression is decreased in the dnaK756 temperature-sensitive mutant, as deduced from the amounts of pulse-labelled MglB synthesized in the mutant and in the parental strain. At 42 °C, there is a reduction in the amount of pulse-labelled MglB in the mutant (31% of the quantity synthesized in the parental strain, in both the precursor and mature form (Fig. 2a, compare lane 1 with lane 2). At 30 °C, in conditions where there is no apparent secretion defect, the amount of pulse-labelled MglB is reduced in the dnaK mutant (35% of the amount synthesized in the parental strain, entirely in the mature form, Fig. 2a, compare lane 3 with lane 4). This decreased expression is selective for MglB: the MglA protein (the cytoplasmic galactose-dependent transport ATPase, which is expressed from the same operon, mglB-AC) is found in similar amounts in the dnaK mutant and in the parental strain, as shown in Fig. 4 where crude extracts from both strains are shown immunoblotted with anti-MglA antibodies. We checked, by pulse-labelling of MglA, immunoprecipitation and electrophoresis, that the synthesis of this protein was unaffected in the dnaK mutant (not shown). The expression of β-galactosidase was also not affected in the dnaK mutant (data not shown).

Unaltered stability of MglB in the dnaK756 mutant

The different levels of MglB expression in the dnaK756 mutant and in the parental strain might be due, in part, to different turnover rates. We measured the half-lives of MglB by labelling exponentially growing cells at 30 °C with [35S]methionine for 2 min, followed by a chase with cold methionine. Samples were withdrawn at various times after the initial labelling, MglB was immunoprecipitated and submitted to electrophoresis. The MglB protein was very stable both in the dnaK mutant and in the wild-type strain (Fig. 5), suggesting that the dnaK
initiation of RNA synthesis. After appropriate time intervals, samples were taken and the RNA was examined by Northern blotting as described in Methods. Thirty micrograms of RNA from the dnaK756 mutant and 15 μg of RNA from the parental strain C600 were loaded into each well. Hybridization was to a labelled probe complementary to residues 398-419 of the mglB mRNA. Hybridization revealed the 1400 nucleotide mglB mRNA. No hybridization was seen when RNA was isolated from cells uninduced for the mgl operon (grown in the absence of fucose and in the presence of glucose).

**Fig. 6.** Decreased stability of the mglB mRNA in the dnaK mutant. Northern blot of mRNA prepared from strain C600 and the dnaK756 mutant. Rifampicin was added to growing cells. RNA was extracted at various times after rifampicin addition and analysed by Northern blotting as described in Methods. Thirty micrograms of RNA from the dnaK756 mutant and 15 μg of RNA from the parental strain C600 were loaded into each well. Hybridization was to a labelled probe complementary to residues 398-419 of the mglB mRNA. Hybridization revealed the 1400 nucleotide mglB mRNA. No hybridization was seen when RNA was isolated from cells uninduced for the mgl operon (grown in the absence of fucose and in the presence of glucose).

**Decreased stability of the mglB mRNA in the dnaK756 mutant**

As described above, the expression of MglB was decreased in the dnaK756 mutant, and the lower MglB expression resulted from a reduction in MglB synthesis since the stability of the protein was unaffected. Furthermore, the reduction in MglB synthesis contrasted with the normal expression of MglA. The high-affinity galactose permease of *E. coli* is encoded by a three-gene operon containing a large intercistronic region with a REP sequence located between the first gene (mglB) and the two distal genes (mglA and mglC) (Hogg et al., 1991). Previous studies of the mglBAC operon have revealed the presence of two mRNA populations (Hogg et al., 1991): one mRNA (mglB mRNA) of approximately 1400 nucleotides corresponds to the 5' region of the operon extending to the intergenic space between mglB and mglA, and a larger mRNA (mglBAC mRNA), about 4300 nucleotides long, represents the entire operon and is present at less than 1/10 of the level of the smaller mRNA population. The following results show that the stability of the mglB mRNA is decreased in the dnaK756 mutant. Cells of the dnaK756 mutant and of the parental strain in exponential phase at 30 °C were treated with rifampicin to inhibit the initiation of RNA synthesis. After appropriate time intervals, samples were taken and the RNA was examined by Northern blotting with a probe complementary to residues 398-419 of mglB (Fig. 6). In the parental strain, the 1400 nucleotide mglB mRNA displayed a half-life of 6 min, similar to that of other mRNAs stabilized by REP sequences such as the malE (Hogg et al., 1991) or the hisP mRNAs (Stern et al., 1988), whereas in the dnaK756 mutant, the half-life of the mglB mRNA was reduced to less than 1 min. The decrease in stability of the mglB mRNA correlated with the decrease in galactose transport activity (Fig. 1a), in the level of MglB (Fig. 1b) and in MglB synthesis (Fig. 2a) in the dnaK mutant. The normal expression and synthesis of MglA in the dnaK mutant suggests that transcription rates of the mglBAC operon, stability of the mglBAC mRNA and translation of the mglA gene are not significantly affected in the mutant [the stability of the mglBAC mRNA could not be determined due to a low hybridization signal with the probe (not shown)]. The decreased MglB expression (Fig. 1b) resulted from a decreased synthesis of the protein (Fig. 2a) and correlated with a decreased stability of the mglB mRNA (Fig. 6). The lesser stability of the mglB mRNA might result from an effect of the dnaK mutation on the stability of the mglB mRNA, perhaps linked to the presence of the REP sequence at the 3' extremity of this mRNA, or from an effect of the dnaK mutation on translation rates of the mglB mRNA with a consequent decrease in its stability (see Discussion).

**DISCUSSION**

In the present report, we show that export and expression of the periplasmic galactose receptor MglB are defective in a dnaK mutant. The defect in export is reflected in an accumulation of pre-MglB in the dnaK756 temperature-sensitive mutant at the restrictive temperature and the defect in expression in reduced MglB synthesis in the mutant (even in conditions where export is not significantly affected). The decreased MglB synthesis correlates with a reduced stability of the mglB mRNA.

Several results suggest that heat shock proteins function as chaperones in protein export: the GroEL/GroES complex appears to be the primary chaperone for the export of β-lactamase (Kusukawa et al., 1989). DnaK and DnaJ are involved in the export of alkaline phosphatase, a SecB-independent protein (Wild et al., 1992), and the maltose receptor MalE and the maltoporin LamR in a SecB null mutant (Wild et al., 1992). It has not been determined whether DnaK (like SecB which functions both as a chaperone and as a pilot protein) helps to target the precursor protein to the translocase or functions solely to maintain the precursor in an export-competent conformation. The present results suggest that DnaK does not substitute for SecB in MglB export, and that it does not eliminate the need for the SecA component of the translocase, thus favouring the hypothesis of an early interaction of the DnaK chaperone with nascent pre-MglB and subsequent transfer of the pre-protein to SecB, SecA and SecY/E.

The synthesis of MglB is reduced in the dnaK mutant (whereas the synthesis of MglA, which is expressed from the same operon, is not affected) and the lower synthesis of MglB correlates with a decreased stability of the mglB mRNA. The mglB mRNA derives from the polycistronic mglBAC mRNA, and is stabilized against 3'-5' exonucleases by a REP sequence located at its 3' extremity (Hogg et al., 1991; Newbury et al., 1987a, b; Stern et al., 1988). It has been shown previously for the malEFG
The features of a RNA-binding protein [its ATPase activity]

MalE deletion of these REP sequences destabilizes the upstream

mRNA by its 3'-located REP sequences seems unlikely, since DnaK does not possess the

features of a RNA-binding protein [its ATPase activity is not affected by nucleic acids but is stimulated by peptides and unfolded proteins (reviewed in Georgopoulos et al., 1994)]. The chaperone might, however, be implicated in the expression of proteins involved in the stabilization of mRNAs by REP sequences (Causton et al., 1994), or in the activity of the multiprotein complex involved in mRNA degradation (Causton et al., 1994; Py et al., 1994; Carppoussis et al., 1994), in the same way that it is implicated in the activity of the multiprotein complex involved in the replication of Lambda phages (Georgopoulos et al., 1994). The implication of DnaK in mRNA stability might be restricted to a select subset of mRNAs, since the heat shock response does not appear to affect the stability of total mRNAs (Henry et al., 1992). The chaperone GroEL has been recently identified as a constituent of an mRNA-protection complex in E. coli (Georgellis et al., 1995).

Alternatively, the decreased stability of the mgfB mRNA in the dnaK mutant might be due to a decreased translation rate of the mgfB mRNA. It has been frequently observed that translation rates can affect the stability of mRNA molecules (Wagner et al., 1994; Yarchuk et al., 1992; Schneider et al., 1978; reviewed in Petersen, 1993), and that synchronization of transcription and translation is important for the stability of nascent bacterial mRNAs (Wagner et al., 1994; Iost & Dreyfus, 1995). DnaK is known to interact with nascent proteins (Hendrick et al., 1993; Gaitanaris et al., 1994), and the chaperone might interact more specifically with exported proteins (particularly with their signal sequence) than with cytoplasmic proteins. Signal sequence mutations can reduce the synthesis of exported proteins (Hall et al., 1983; Puziss et al., 1989, 1992), and in some cases this reduction occurs independently of an export defect (Puziss et al., 1992). There might be some coupling between a DnaK-dependent folding of MglB and translation rates of the mgfB mRNA, leading to a decreased stability of the message in a dnaK mutant. A cooperation between the translation machinery and yeast hsp70s of the Ssb class has been reported (Nelson et al., 1992), and DnaK appears to regulate synthesis of the heat shock Sigma factor σ22 at the translational level by interacting with a distinct region of the nascent protein (Nagai et al., 1994). Several results suggest that in E. coli, 4-5S RNA regulates translation to allow proteins to fold properly, and that the Ffh/4-5S RNA complex couples translation of exported proteins to membrane translocation (Luirink & Dobberstein, 1994; Brown, 1991; Phillips & Silhavy, 1992). The DnaK/ hsp70 chaperones might also be involved in such coupling processes associated with protein folding and protein export.

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