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Identification and characterization of GroEL and DnaK homologues in *Thiobacillus ferrooxidans*

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1. SUMMARY

The major heat shock proteins from *Thiobacillus ferrooxidans* were identified as DnaK and GroEL equivalents by Western blotting and analysis of the N-terminal amino acid sequence of spots isolated from dried 2-D polyacrylamide electrophoresis gels. The *T. ferrooxidans* chaperonins showed 70% and 80% identity with the *Escherichia coli* GroEL and DnaK, respectively. By using electrophoresis with a transverse pore gradient of cross-linked polyacrylamide and non-denaturing conditions followed by Western blotting, we found that the GroEL proteins from both bacteria formed a 14-mer, whereas *E. coli* DnaK protein existed partially as a dimer and the *T. ferrooxidans* DnaK-equivalent showed only a monomeric nature under our experimental conditions.

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2. INTRODUCTION

During bacterial leaching of ores, microorganisms are subjected to different kinds of environmental stress such as temperature changes, presence of some toxic heavy metals or pH changes which normally take place on bioleaching operations and that may affect the activity of the bacteria [1,2]. In response to these stressing conditions, bacteria reduce the genetic expression of most normal cellular proteins, inducing at the same time a transient overproduction of the so-called stress proteins [3,4]. We have previously reported the existence of a heat shock response in *Thiobacillus ferrooxidans* [5,6] and *Sulfolobus acidocaldarius* [6] and a pH-stress response in *T. ferrooxidans* [7].

In *Escherichia coli*, some of the major heat shock proteins are GroEL and DnaK proteins, which are highly conserved in most organisms [4,8,9]. These polypeptides have been considered to be molecular chaperones and that their function in the cell is to mediate the correct assembly of some oligomeric proteins from their subunits

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[9]. In the present report, we extended our studies of the heat shock response in the acidophilic chemolithotropic *T. ferrooxidans* and identified and characterized the proteins equivalent to DnaK and GroEL in this industrially important bacteria.

3. MATERIALS AND METHODS

3.1. Organisms and growth conditions

T. ferrooxidans ATCC 19859 was grown at pH 1.5 and at 30°C in a modified 9K liquid medium [6,7] and *E. coli* RP 437 was grown at 37°C in the TYE culture medium [10].

3.2. Preparation of cell-free extracts

T. ferrooxidans cells were harvested and washed twice by centrifugation with diluted H_2SO_4 at pH 1.5 and once with 0.5 M sodium citrate, pH 7. Finally, cells were resuspended in 10 mM Tris · HCl, pH 7.4, 5 mM $MgCl_2$ and 0.1% 2-mercaptoethanol and subjected to sonic oscillation (four times for 30 s at 200 W in a 4710 Cole Palmer ultrasonic homogenizer). The cell-free extract was finally obtained by centrifugation at $12000 \times g$ during 5 min.

3.3. Non-denaturing and two-dimensional PAGE

Non-denaturing PAGE was carried out in mini-slabs according to Retamal and Babul [11], except that the polyacrylamide solutions were prepared according to Laemmli [12], but without the inclusion of SDS. For 2-D PAGE, we employed the 2-D NEPHGE procedure described by O'Farrell [14] as before [7]. Protein bands were visualized by Coomassie brilliant blue staining or after transfer of the proteins to a nitrocellulose membrane followed by Western immunoblotting [13]. The polyclonal antisera employed and their dilutions were-anti DnaK (1 : 1000) and anti GroEL (1 : 1000) from *E. coli*. Development was done by using anti-rabbit Ig, biotinylated species-specific whole antibody (from donkey) and streptavidin-biotinylated horseradish peroxidase complex. The substrate was 4-chloro-1-naphtol and was used according to the manufacturer's instructions (Amersham International).

3.4. Microsequencing of proteins

The protein of interest was recovered from Coomassie brilliant blue-stained and heat-dried two-dimensional gels by excising the protein spots with a minimum of polyacrylamide and then submerging them in 50 mM boric acid (adjusted to pH 8.0 with NaOH) containing 0.1% SDS as described by Bauw et al. [15]. After 2 h of rehydration, the swollen gel pieces (usually five spots of the same protein) were taken up with tweezers and placed in a gel slot of a new SDS-slab gel [15]. Gel electrophoresis was carried out as described before [6,7].

After the run, the proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes and stained with Coomassie brilliant blue [16]. The membrane piece carrying the protein was excised and was subjected to microsequencing by the Protein Chemistry Laboratory at Hoffmann-La Roche Inc., Nutley, NJ.

4. RESULTS AND DISCUSSION

4.1. Identification of T. ferrooxidans DnaK and GroEL homologues in 2-D gels

We have previously identified DnaK and GroEL from *T. ferrooxidans* by using *E. coli* anti DnaK and anti GroEL antibodies (Alvarez, Seeger and Jerez, unpublished). Figure 1 shows the locations of the corresponding spots after separation of total proteins from *T. ferrooxidans* by 2-D NEPHGE. From this gel, the M_r values can be estimated as 56 300 for GroEL and 67 600 for DnaK.

4.2. Amino-terminal end microsequence of T. ferrooxidans DnaK and GroEL equivalents

Seven DnaK and four GroEL spots similar to the ones seen in Fig. 1 were excised from the dried gels, and after transfer of the pooled and concentrated proteins to a PVDF membrane they were subjected to microsequencing of their N-terminal regions. It is clear that over the segments sequenced there is a high degree of homology (16 out of 23 are identical) between the *T. ferrooxidans* GroEL and the *E. coli* GroEL (Fig. 2). Somewhat lower homologies were also ob-

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Fig. 1. Identification of DnaK and GroEL from *T. ferrooxidans* in a 2-D gel. Total *T. ferrooxidans* proteins were separated by 2-D NEPHGE. The spots indicated were excised from the gels for further analysis. Numbers indicate the molecular masses in kDa.

T. ferrooxidans	PAKQVAFAEHAREKMLRGVNVLA
E. coli	MAAKDVKFGNDARVKMLRGVNVLA
M. tuberculosis	MAKTIAYDEEARRGLERGLNSLA
M. leprae	MAKTIAYDEEARRGLERGLNALA
R. sphaeroides	AAKDVKFDTDARDXMLRGV----

Fig. 2. N-terminal amino-acid sequences of the *T. ferrooxidans* GroEL and DnaK homologues compared with several bacteria. The top group of sequences correspond to GroEL homologues from *T. ferrooxidans*, *E. coli* [20], *Mycobacterium tuberculosis* [21], *Mycobacterium leprae* [22] and *Rhodobacter sphaeroides* [23]. The bottom group of sequences represent DnaK equivalents from *T. ferrooxidans*, *E. coli* [24], *Bacillus subtilis* [25], *Caulobacter crescentus* [26] and *Mycobacterium bovis* [27].

Fig. 3. Separation of DnaK and GroEL proteins under non-denaturing conditions. Pure *E. coli* GroEL (**a**), and DnaK (**d**) or crude cell-free extracts from *E. coli* (**b, e**) or from *T. ferrooxidans* (**c, f**) were separated by non-denaturing polyacrylamide electrophoresis at the indicated polyacrylamide gradient concentrations, and immunoblotted with *E. coli* anti GroEL (**a, b, c**) or anti DnaK (**d, e, f**). Arrows indicate a putative *E. coli* DnaK dimer.

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served when the *T. ferrooxidans* protein was compared to GroEL equivalents from other microorganisms (Fig. 2). On the other hand, the DnaK protein from *T. ferrooxidans* also showed a high degree of identity with the *E. coli* DnaK (15 out of 19 were identical) and with other bacterial 70 kDa hsps (Fig. 2).

4.3. Oligomeric nature of E. coli and T. ferrooxidans chaperonins

The GroEL protein from *E. coli* is normally present in cells as a tetradecameric complex [17]. To study the possible oligomeric forms of the *T. ferrooxidans* chaperonins, we employed non-denaturing polyacrylamide electrophoresis with the use of a gel with a transverse pore gradient of cross-linked polyacrylamide. This method is very convenient since it eliminates the need for several gels of different acrylamide concentrations [11]. Figure 3 shows the results obtained with this method followed by Western blotting to identify the proteins. We first determined the slope of the relative migration (R_f) of marker proteins and the *E. coli* and *T. ferrooxidans* GroEl and DnaK proteins at the different acrylamide concentrations. From these values, we could calculate their M_r values (Fig. 4). *E. coli* GroEL (Fig. 3a) showed a M_r of 814 000, which corresponded well with a tetradecameric complex in which the monomeric protein has a M_r of about 60 000. An equivalent result was obtained for *E. coli* when a crude cell-free extract was employed instead of the pure protein (Fig. 3b). *T. ferrooxidans* GroEL present in a cell-free extract (Fig. 3c), showed a M_r of 804 000. According to its monomeric size (Fig. 1), the *T. ferrooxidans* chaperone could also have a tetradecameric structure. In general, no monomeric form for GroEL was seen under the electrophoretic conditions used, in agreement with the very high stability for the oligomeric form of this protein [18]. Hsp70 protein is purified predominantly as a monomer. However, the functional form of hsp70 is believed to be a multimer of unknown size [4]. To test this with the *E. coli* hsp70, we ran pure *E. coli* DnaK protein and a cell-free extract in the non-denaturing PAGE system (Fig 3d, e, respectively). The arrows indicate the formation of a possible DnaK dimer with a

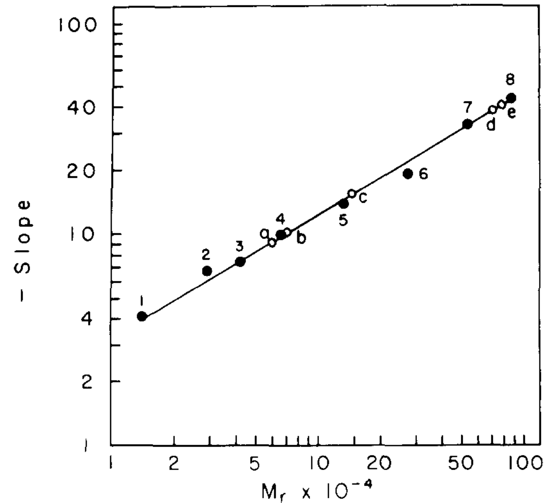

Fig. 4. Determination of molecular masses of DnaK and GroEL proteins. Plot of the slopes of the relative migration (R_f) of marker proteins and the different *E. coli* and *T. ferrooxidans* GroEL and DnaK proteins separated in Fig. 3, at the different acrylamide concentrations. The proteins and their M_r were (1) lactalbumin, 14000; (2) carbonic anhydrase, 29000; (3) ovalbumin, 45000; (4) bovine serum albumin, 66000 (monomer); (5) bovine serum albumin, 132000 (dimer); (6) urease, 272000 (trimer); (7) urease, 545000 (hexamer); (8) ferritin, 880000 (dimer); (a) *E. coli* DnaK (monomer); (b) *T. ferrooxidans* DnaK (monomer); (c) *E. coli* DnaK (dimer); (d) *E. coli* GroEL (tetradecamer); (e) *T. ferrooxidans* GroEL (tetradecamer).

M_r value of 149 000 in *E. coli*. When the possible dimer band was excised from the nitrocelullose membrane, and was rerun on an SDS-PAGE system, only monomeric (67 600) DnaK was obtained (data not shown). It has been shown that a cytosolic hsp70 can form dimers, trimers and even larger oligomeric species in vitro [19]. To our knowledge, the data reported here form the first experimental evidence indicating that *E. coli* DnaK protein may form dimers. Both monomeric and dimeric forms of *E. coli* DnaK were present, suggesting a weaker interaction between the monomeric forms of this polypeptide compared with the GroEL oligomer. When a cell-free extract from *T. ferrooxidans* was tested (Fig. 3f), only monomeric DnaK was observed. If dimers exist, perhaps they were not seen due to a lack of sensitivity of the immunological reaction of the *T. ferrooxidans* protein with *E. coli* anti DnaK.

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