Chapter 3.
Results
3.1. Sequence analysis of HspR

Phylogenetic analysis

HspR like repressors are found in a wide variety of bacteria, from both Gram-positive and Gram-negative. In order to understand the evolutionary relationships between HspRs of diverse bacteria a phylogenetic analysis was performed. Alignment of HspR sequences from various Eubacteria using ClustalW reveals that the N-terminal half of the protein, which includes the winged Helix-turn-Helix motif DNA binding domain (HTH-W1-W2), is highly conserved (Fig. 22A). In contrast the sequence in the C-terminal region is variable. Phylogenetic analysis based on the conserved sequences (aligned positions 50-130) revealed that there are several distinct clades of HspR (Fig. 22B). The HspRs of Eubacteria belonging to the phylum Thermus-Deinococcus form a distinct and possibly ancient clade. These HspRs possess a duplication of their HspR domains designated as 1 and 2. HspRs of more modern Eubacteria, the Campylobacterales, C. jejune and H. pylori form a different clade. Interestingly these HspRs appear to be closely related to the HspR of Aquifex aeolicus, hyperthermophillic bacteria, belonging to the ancient phylum Aquificae. This observation suggests that the Campylobacterales may have acquired their hspR orthologs from ancient thermophillic bacteria through horizontal transfer. The third clade comprises HspRs derived from bacteria belonging to the phylum Actinobacteria. Within this clade a subgroup of HspRs derived from Actinomycetales form a distinct branch. The HspRs of this group are characterized by the presence of a conserved LVVW motif at the C-terminal region of HspR (Fig. 22A, boxed region). The HspR from Propionibacterium is clearly an exception in that although it is derived from an Actinomycetale yet it clusters with the Bifidobacterale HspR (Fig. 22B). Also it does not possess the conserved LVVW motif (Fig. 22A). The phylogenetic analysis was performed only with a representative set of Actinomycetale HspRs. The sequences of many other such HspRs are available in the databases. With a few exceptions such as that of Propionibacterium most Actinomycetale HspRs possess this motif.

A general feature of the Actinomycetale HspRs is that they are prone to aggregation and therefore they can be purified only under denaturing conditions (11). Since the
Figure 22. Sequence comparison of HspRs. A) ClustalW alignment (Gap opening and extension penalties of 10 and 0.1 respectively) of representative HspRs derived from a broad spectrum of Eubacteria using MEGA 4.0. The Similar/Identical residues (PAM matrix) were colored using Bioedit tools. The motif conserved in most of the Actinomycetales is boxed and blown up for details. The arrow indicates the position at which the WT protein was truncated to obtain the AC mutant. The positions of aligned charged amino acid residues flanking the ‘LVW’ core are highlighted with red dots. The Helix-turn-Helix (HTH) DNA binding motif and the associated wing domains (W1 and W2) are indicated on the top. The abbreviations are as follows. Mtb, Mycobacterium tuberculosis; Sco, Streptomyces coelicolor; Twh, Tropheryma whippeli; Gal, Gordonia alkanivorans; Cgl, Corynebacterium glutamicum; Nfa, Nocardia farcinica; Pac, Propionibacterium acnes; Bbe, Bifidobacterium breve; Cje, Campylobacter jejuni; Hpy, Helicobacter pylori; Aae, Aquifex aeolicus; Dra, Deinococcus radiodurans; Dge, Deinococcus geothermalis; Taq, Thermus aquaticus. The HspRs of Dra, Dge and Taq have duplicated HspR domains marked as 1 and 2 (e.g. Dra1 and Dra2). (B) Evolutionary relationships between 17 HspR taxa. The conserved winged HTH (HTH-W1-W2) region of the alignment (A) was used for tree construction using the neighbor-joining method (97). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches.
motif LVVVW, conserved in most of the Actinomycetale HspRs, is rich in hydrophobic amino acids, one of which is tryptophan, considered as the most hydrophobic of all amino acids (76), therefore it is possible that the C-terminal tail may have a role to play in promoting the aggregation of these HspRs. Moreover it has been reported that the solubility and binding activity of HspR depends to a large extent on the presence of DnaK. DnaK is known to bind to the hydrophobic peptides particularly those which are leucine rich and flanked by positively charged amino acids (91). The LVVVW motif, flanked by positively charged amino acids lysine and arginine (Fig. 22A boxed region, indicated by dots) strongly resembles with the consensus DnaK binding motif. Hence it may be proposed that the C-terminal tail of HspR plays an important role in DnaK binding. In order to obtain insight into the interaction between DnaK and HspR, in silico modeling experiments were performed.

Modeling study

To model the interaction between HspR and DnaK, it became necessary to build their three dimensional structures. Since no crystal structure of HspR has yet been reported, homology modeling of HspR was attempted. Conserved domain search with the amino acid sequence of HspR of M. tuberculosis revealed that HspR belongs to the MerR super-family. Crystal structures have been reported for several MerR family proteins. To identify MerR family members, which may be structurally related to HspR, a Blast search was performed using Protein Data Bank (PDB) database and several structural homologues of HspR were identified. These includes several MerR family proteins e.g. SoxR, Mtan, Tipal etc. Although the overall sequence identity between HspR of M. tuberculosis and these proteins are only 33-38%, the predicted structure of HspR is similar to these proteins in the region that includes the N-terminal DNA binding domain (15 to 90 of HspR). Among these proteins the DNA binding site of SoxR is very similar to that of HspR. Both recognize a self-complementary palindromic sequence. It suggests that the three dimensional structural orientation of HspR protein must be similar to that of SoxR to attain the same DNA binding conformation. Therefore the crystal structure of SoxR (PDB id. 2ZHH, chain A) was used as template for homology modeling of HspR. Modeling shows that HspR possesses a winged Helix-Turn-Helix (H-T-H) like structure characteristics of MerR family proteins (8).
contains three domains: H-T-H, wing I and wing II. The H-T-H domain consists of helix1 (α1) and helix2 (α2) (Fig. 23, A and B). The wing I includes two beta strands (β2 and β3) and the connecting loops. Wing II is the combination of helix3 (α3) and helix4 (α4).

Various crystal structures of DnaK, unbound or bound with several peptides have been reported so far. NCBI Blast search shows 99% identity of DnaK from *M. tuberculosis* with the protein sequence of DnaK from *E. coli*. So the homology modeling of DnaK was done using the crystal structure of DnaK of *E. coli* (PDB id 1DKX) as template.
Docking experiment was performed in which HspR was docked to DnaK to determine the preferred DnaK binding site on HspR. Here we used rigid body docking by refining a model of a complex structure by optimizing the separation between the interactors but keeping their relative orientations fixed. The relative orientations of the interacting partners in the modeling were allowed to vary, but the internal geometry of each of the partners was held fixed. The experiment generated 10 good models, and the best model was selected on the basis of our hypothesis. Fig. 23C shows the binding mode of DnaK with HspR. As evident from Fig. 23C the major interactions are occurring between the N-terminal part of substrate binding domain (SBD) of DnaK and C-terminal tail of HspR. In addition to the tail region, the helix 3 of HspR takes a major role in interacting with loop 5, 6 and helix E of SBD. The helices E and D of DnaK together act as a lid to support the HspR-DnaK complex. On further inspection it has been revealed that Val113 interacts with Thr390 of DnaK; Thr117 interacts with Thr390 and Thr391 of DnaK and Val120 interacts with Thr391, Val368 and Leu366 at N-terminal SBD of DnaK. Interestingly all the above-mentioned interactions are highly hydrophobic in nature. Accessible surface area determination (Table 17) indicates that the N-terminal part of SBD of DnaK and C-terminal tail of HspR are involved in major interactions. Amongst all residues in HspR Val113, Val120 at the C-terminal tail of HspR lose the maximum surface area on binding indicating that these residues may strongly interact with DnaK. V120 residue is within the conserved LVVW motif, which is identified as the primary DnaK binding site. This indicates that this region is primarily involved in the interaction with DnaK. Apart from this motif, there are some other residues which lose their ASA in considerable extents. This suggests that there are other secondary sites involved in DnaK binding.

Docking experiments predict that there are at least two regions of DnaK which are involved in DnaK-HspR interactions. The most critical contacts appear to involve the Substrate binding domain of DnaK and the C-terminal tail of HspR. The substrate
binding site of DnaK is a β sandwich structure (Fig. 24) comprising eight β strands with loops connecting one strand with the other. The peptidic substrates reside within a pocket created by the sandwich structure and the loops joining them. The loops 1,2 and 3,4 are particularly involved in caging the peptide. In this case HspR appears to make a significant contact with β3 and Loop 5,6 of DnaK which are located on the outside of the peptide binding pocket (Fig. 24). The results of these modeling studies show that a) there are multiple sites in HspR which are involved in the interaction with DnaK and b) maximal interaction appears to involve val113 and val120 which are located in the C-terminal region of HspR.

<table>
<thead>
<tr>
<th>Residues of Location in HspR</th>
<th>ASA (HspR)</th>
<th>ASA (HspR-DnaK)</th>
<th>ΔASA</th>
<th>Interacting parts of DnaK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu20 Helix1</td>
<td>115.5</td>
<td>54.1</td>
<td>61.4</td>
<td>Loop5,6</td>
</tr>
<tr>
<td>Tyr50 Helix3</td>
<td>98.8</td>
<td>27.6</td>
<td>71.2</td>
<td>HelixE</td>
</tr>
<tr>
<td>Ser51 Helix3</td>
<td>62.1</td>
<td>0.1</td>
<td>62.0</td>
<td>HelixE</td>
</tr>
<tr>
<td>Leu52 Helix3</td>
<td>55.4</td>
<td>6.1</td>
<td>49.3</td>
<td>HelixE</td>
</tr>
<tr>
<td>Leu58 Helix3</td>
<td>80.7</td>
<td>10.4</td>
<td>70.3</td>
<td>Loop5,6</td>
</tr>
<tr>
<td>Val61 Helix3</td>
<td>84.1</td>
<td>11.2</td>
<td>72.9</td>
<td>Loop5,6</td>
</tr>
<tr>
<td>Gln62 Helix3</td>
<td>59.9</td>
<td>19.9</td>
<td>40.0</td>
<td>Loop5,6</td>
</tr>
<tr>
<td>Arg91 Helix5</td>
<td>50.4</td>
<td>4.7</td>
<td>45.7</td>
<td>HelixD</td>
</tr>
<tr>
<td>Leu92 Helix5</td>
<td>116.1</td>
<td>37.3</td>
<td>78.8</td>
<td>HelixD</td>
</tr>
<tr>
<td>Arg103 Helix5</td>
<td>143.2</td>
<td>85.6</td>
<td>57.6</td>
<td>HelixB</td>
</tr>
<tr>
<td>Gln106 Helix5</td>
<td>100.3</td>
<td>26.1</td>
<td>74.2</td>
<td>Loop2,3</td>
</tr>
<tr>
<td>Val110 Helix5</td>
<td>94.2</td>
<td>38.8</td>
<td>55.4</td>
<td>Loop2,3</td>
</tr>
<tr>
<td>Val113 Helix5</td>
<td>99.3</td>
<td>1.8</td>
<td>97.5</td>
<td>Beta 3</td>
</tr>
<tr>
<td>Thr117 Helix5</td>
<td>91.4</td>
<td>37.8</td>
<td>53.6</td>
<td>Beta 3</td>
</tr>
<tr>
<td>Val120 Helix5</td>
<td>120.3</td>
<td>17.8</td>
<td>102.5</td>
<td>Beta3, N-terminus of SBD domain</td>
</tr>
</tbody>
</table>

Table 17 | The accessible surface area (16) of HspR, DnaK and HspR-DnaK complex were calculated using GETAREA. The differences in the ASA (ΔASA) between HspR alone and HspR-DnaK complex were considered as indicators of major interactions.
3.2. Renaturation of HspR

From sequence analysis and modeling study, the LVWW motif was identified as the possible primary target site for binding with DnaK. It was also suspected that the LVWW motif may be responsible for the tendency of HspRs derived from Actinomycetales to form aggregates as reported earlier (9, 27, 126). To test these possibilities HspR was truncated by changing the codon corresponding to Leu119 to a stop codon (arrow in Fig. 22A) and the resulting protein was the C-terminal tail deletion mutant named, HspR-ΔC. The wild type and mutant proteins were then synthesized in *E. coli* at a high level using inducible expression systems. Purification of HspR-WT was then attempted under both denaturation and native conditions. The results show that wild type HspR is synthesized at high levels following IPTG induction and can be purified to near homogeneity if purified under denaturing conditions (Fig. 25A). Similar pattern was observed in case of HspR-ΔC when it was purified under denaturing condition (data not shown). To confirm the identities of the purified proteins Peptide mass fingerprinting was performed (Supplementary information: page 89). Unlike in the case of denaturing conditions, when purification was attempted under native conditions presence of several contaminating bands were observed in case of HspR-WT (Fig. 25B; lanes 2 to 5). At least one band could be easily identified as DnaK, based on its mobility. DnaK has been reported to co-purify with HspR earlier also (11) and therefore this result was expected. However the interesting observation however was that, in contrast to WT, in case of the mutant protein, the level of DnaK

![Figure 25. Purification of HspR.](image-url)
co-purifying with HspR-ΔC was almost non-existent (Fig. 25B; lanes 6 to 9). This indicates that the C-terminal tail of HspR may be involved in the interaction with DnaK.

**Renaturation of HspR wild type and its C-terminal deletion mutant.** To test whether the C-terminal tail deletion led to any improvement in *in vitro* refolding efficacy of HspR, attempts were made to re-nature HspR-WT and HspR-ΔC from the denatured state, by stepwise removal of the denaturant, urea, through dialysis. HspR-WT renatured by this procedure aggregated resulting in poor recovery of the soluble form, most of it (80%) being present in the insoluble fraction (Fig. 26; lanes 3 and 5 compared to 1). HspR-ΔC on the other hand remained soluble. Most of the protein (~87.5%) was recovered in the soluble supernatant whereas as a small amount (~12.5%) was found to be present in pellet fraction (Fig. 26; lanes 4 and 6 compared to 2). The results therefore indicate a significant increase in the solubility of HspR following C-terminal deletion.

**Renaturation of HspR-Wt using Size Exclusion Chromatography.** The results presented in the previous section indicate that HspR-WT cannot be refolded using the method of dialysis. Hence an alternative method was attempted in which the

![Figure 27.](image)
denaturant was removed not by dialysis, but by Size Exclusion Chromatography. This technique has been used earlier to refold proteins that are difficult to renature (5). The experiment was performed by loading the 1 ml of the protein dissolved in 8M urea containing buffer (1mg/ml) onto a SEC column (exclusion limit 200 kDa), followed by elution. Since the molecular weight of urea is much less compared to HspR, its mobility is expected to be retarded to such an extent that it will be separated from the protein as it passes through the gel filtration column. The results show that the major part of the protein eluted as a single peak corresponding to the HspR monomer (Fig. 27A and B). The secondary structure of HspR present in the pooled peak fractions was investigated using CD spectroscopy. For the sake of comparison, HspR-ΔC renatured by dialysis was also subjected to CD spectroscopy. From the spectra (Fig. 27C and D) the helical contents of both the re-natured proteins were calculated to be about 33%. The results indicate that the WT protein can be renatured effectively by performing SEC, and that deletion of the C-terminal region did not result in any gross changes in the structure of HspR.

**DNA binding activities of renatured Wt and mutant proteins.** The ability of the purified HspR wild type and mutant to bind to the operator was then tested using EMSA. Since these proteins cannot be obtained in a highly purified form, using native conditions therefore they were isolated under denaturing conditions and then

![Figure 28. DNA binding activity of HspR. The WT protein renatured by SEC (WT<sup>sec</sup>) and ΔC by the dialysis method (ΔC<sup>d</sup>) were used in EMSA experiments using the labeled HspR binding site as probe. (A) EMSA was performed with increasing concentrations (0.5, 1, 1.5 and 2 μM) of renatured proteins in a final reaction volume of 30 μl. The complexes are marked as C1 and C2. (B) and (C), Competition binding assays performed with WT and ΔC proteins respectively, using the indicated molar excess (125-500) of competitor either self (Specific) or an unrelated (Nonspecific) double stranded oligonucleotide representing the origin region of plasmid pAL5000. In the lanes marked (-) no protein was added. The band corresponding to the free probe is marked F.](image-url)
renatured, by removing the denaturant. DNA binding assays were performed using HspR-WT and HspR-ΔC renatured by SEC (WT<sup>35</sup>) and dialysis (ΔC<sup>39</sup>) respectively. The probe used was a 32P labeled 110 bp DNA segment encompassing the HspR binding sites located upstream of the <i>M. tuberculosis</i> dnaKJE-hspR operon (-128 to -38). Concentration dependent increase in binding of HspR-WT and HspR-ΔC to the probe was observed. At the lower concentration C1 is formed, but as the concentration increased a second complex C2 appeared (Fig. 28A). To obtain an idea about specificities, competition binding experiments were performed using molar excess of either self (specific) and or an unrelated (non-specific) competitor, a 150 bp fragment derived from the mycobacterial plasmid pAL5000 origin of replication (4) (Figs. 28B and C). The resulting competition experiments revealed that the complexes were competed out in a dose dependent manner only by the specific but not the nonspecific competitor. This indicates that the complexes were specific. Moreover the rate of competition by self-competitor was almost the same for both WT and ΔC. This indicates that the affinity of HspR for its target site did not change significantly following removal of the C-terminal region.

### DnaK assisted renaturation of HspR

The previous experiments indicate that renatured HspR can bind to the operator sites in the absence of DnaK. Hence the requirement of DnaK is not obligatory as thought earlier (11). However DnaK, being a

![Figure 29. DNA binding activity of HspR renatured with DnaK assistance. (A) HspRs either WT or ΔC denatured using 8M urea was diluted 100 times into renaturation buffer containing 0.5 mM ATP in presence or absence of 2 μM DnaK. Following pre-incubation labeled probe was added. (B) Competitive EMSA was performed to examine the specificity of the C4 complex formed in case of HspR-WT, using molar excess of self (specific) or an unrelated (nonspecific) competitor DNA as indicated on the top. (C) Antibody-supershift EMSA, using either Pre-immune sera (lane marked PI) or anti-DnaK immune sera (lane marked I). Lanes marked (-) in B and C contain no protein. The position of the band corresponding to the free probe (F) is shown in each autoradiogram.](image-url)
chaperone, may have a role to play in activating HspR's DNA binding activity under conditions in which renaturation of HspR is inefficient. To investigate such a possibility 8M urea-denatured HspR was renatured by rapid dilution into urea free buffer. To assess whether the protein could renature under these conditions, EMSA experiments were performed. The results show that when denatured HspR-WT was diluted, no binding activity could be recovered indicating that HspR-WT was unable to regain activity (Fig. 29A; lane 2). On the other hand when denatured HspR-ΔC was similarly diluted, formation of complexes C1 and C2 was observed (Fig. 29A; lane 4). This indicated that ΔC, but not WT, could be re-natured by dilution into denaturant free buffer, without the assistance of DnaK. When denatured HspR-WT was diluted into DnaK containing renaturation buffer DNA binding activity was observed but the mobility of the complex formed (C4) did not match that of either C1-C2 or C3. It migrated slower than the rest (Fig. 29A; lane 3). Unlike HspR-WT, denatured HspR-ΔC did not form C4 in the presence of DnaK. The predominant complexes were C1 and C2 (Fig. 29A; lane 5). However the minor complex C3 was also found to be present.

Considering that the mobility of complex C4 formed by HspR-WT, was distinctly different from that of C1, C2 and C3 complexes which are formed independent of DnaK, the question arose whether this complex is specific. A competition DNA binding assay was thus performed. The results showed that C4 was indeed competed out by self competitor (Fig. 29B; lanes 2 to 6), but not by an unrelated competitor (Fig. 29B; lanes 7 to 11), indicating that it is specific. Since C4 was formed only in the presence of DnaK, therefore it was necessary to examine whether the added DnaK became a part of complex C4. Supershift assay was performed using antisera against DnaK. The corresponding pre-immune sera was used as a control. The results show that in the presence of Anti-DnaK sera a supershift was observed (Fig. 29C; lane 4). The pre-immune sera had no effect (Fig. 29C; lane 3). The results of these experiments confirm that DnaK has a role to play in activating HspR under conditions where HspR cannot renature easily. However in such cases DnaK itself becomes a part of the complex. No such constrain was observed in case of HspR-ΔC which renatured efficiently in the absence of DnaK. Moreover even, in the presence of DnaK, there was no indication of the formation of C4 type HspR-DnaK high molecular weight complex.
Protective action of DnaK against thermal denaturation of HspR. In the previous sections the renaturation efficiency of denatured HspR was tested under different conditions. It was found that given appropriate conditions HspR can renature without the help of DnaK. The question addressed in this section is whether DnaK can protect against the denaturation of HspR which has been renatured by the method of SEC or dialysis. HspR is a regulator of the heat shock response, and hence its thermal stability is likely to play a key role in its ability to function as repressor. To test this hypothesis that HspR is thermolabile the operator binding activity of HspR was examined at 25°C (Room temperature) and at 42°C (Heat Shock temperature). The results showed that as expected the binding activity of HspR was completely abolished under heat shock conditions (Fig. 30; lane 5). In sharp contrast, HspR-ΔC was stable towards heat shock. The presence or absence of DnaK or Hsp16.3 made no difference (Fig. 30; lanes 8-13). The question that was raised next was whether DnaK had any protective effect. The results show that in the presence of DnaK no significant loss of HspR’s origin binding activity was observed (Fig. 30, lane 6). The predominant complexes C1 and C2, and the minor complex C3 were formed. The addition of another chaperone Hsp16.3 also led to some protection (Fig. 30; lane 7). These results indicate DnaK can protect against the thermal denaturation of HspR. However, unlike in case of assisted renaturation, DnaK did not form a complex with HspR in this case. The other interesting result is that the deletion mutant, HspR-ΔC, remained highly stable even under heat shock conditions.
3.3. DnaK-HspR interactions

The results presented in the previous section indicate that the C-terminal deletion mutant of HspR possesses renaturation properties that are distinct from that of wild type. Moreover, it appeared that this deletion led to a loss of DnaK requirement. To obtain an insight into how this deletion may have affected the DnaK dependence properties of HspR, in vitro and in vivo experiments were performed. In the in vitro experiments the affinity of the wild type and mutant HspR for DnaK were compared, whereas in the in vivo experiments the effect of the deletion on the ability of HspR to function as a repressor was tested.

Affinity comparison

In order to assess the affinity of DnaK for HspR-WT and ΔC, direct binding assays were performed using a Microtiter plate based method. In this assay 6X His tagged HspR either WT or ΔC was allowed to bind in solution to a tag free version of mycobacterial DnaK. The DnaK-HspR complexes formed were immobilized on to the wells of a Ni-NTA HisSorb plate in duplicate. One well of each duplicate set was probed with anti-HspR sera and the other with anti-DnaK. The results show that DnaK binding was maximum in case of denatured HspR-WT (Fig. 31A). In case of renatured HspR-WT binding was only marginally higher than background levels. That this difference is not due to variations in the amount of HspR present in the assay system, is evident from the observation that there were only minor differences in the corresponding A_{405} (HspR) values (Fig. 31B). As in case of the renatured WT protein,

(A) (B) (C)

Figure 31. ELISA to study interaction between DnaK and HspR, either WT or ΔC. Mean (A_{405}) values derived from five replicate ELISA experiments using antisera against DnaK (A) or HspR (B) are shown. (C) Means of the Absorbance ratios between wells probed with anti-DnaK and anti-HspR respectively. Error bars signify ± SEM (Standard Error of Mean). White and shaded bars correspond to denatured and renatured samples respectively. Striped bar shows the results of a mock experiment in which HspR was missing.
the level of DnaK binding to renatured HspR-ΔC was low, but the interesting observations was that unlike in case of WT, denatured HspR-ΔC did not show any significant binding to DnaK. The observed differences became more pronounced if the minor fluctuations in the amount of HspR bound to the support were taken into account and the data normalized accordingly (Fig. 31C). Such normalization was done by finding the ratios (Absorbance ratio) between the respective A$_{405}$ values for DnaK and HspR. The major conclusions from this experiment are: a) the hydrophobic C-terminal tail plays an important role in the ability of HspR to interact with the DnaK and b) presence of the tail is not enough, it has to be present in the exposed (denatured) state for productive interaction with DnaK.

**Comparison of repression efficiencies**

In order to test whether deletion of the C-terminal tail affects the ability of HspR to act as a repressor an experiment was designed using the surrogate host *E. coli*. Reporter activity from pSDTD2 or the control plasmid pSD5S30B, which expresses the reporter gene *lacZ* from the unrelated S30 promoter, not known to be heat inducible, was assessed in the absence or presence of HspR synthesized from the co-transformed plasmids either pTDR30 (for HspR-WT) or pTDR30-1 (for HspR ΔC). Following induction of HspR synthesis the samples were divided into two parts, one part received no heat shock while the other did (Fig. 32A and B respectively). The results showed that the reporter activities from both pSDTD2 and pSD5S30B were higher compared to that of the promoter less vector pSD5B. Hence both promoters were active in *E. coli*. The activity of pSDTD2 was however more than pSD5S30B. When HspR was supplied in trans (pSDTD2+WT), partial repression of *lacZ* expression...
was observed in the absence of heat shock (Fig. 32A), but for HspR-ΔC (pSDTD2+ΔC) repression was near complete. Upon heat shock (Fig. 32B), de-repression from the dnaKJE-hspR promoter was clearly evident in the case where repression was mediated by the WT repressor (pSDTD2+WT), but no such effect was observed in the case of ΔC (pSDTD2+ΔC). What emerges from this experiment is that HspR-ΔC functions as a stronger repressor \textit{in vivo} as compared to WT. Moreover unlike in case of WT heat shock could not relieve the repression mediated by HspR-ΔC. These effects are clearly specific to the dnaKJE-hspR operon promoter as expression from the control plasmid pSD5S30B, remained almost unaffected in the presence of HspR, either WT or ΔC. Small differences if any were found to be statistically insignificant.