I. Introduction

In the evolution from single cell organisms to higher organisms, cellular differentiation and multicellular morphorgenesis of unicellular creatures occupy the early step. It provides the first clue to address the problem how genes control development.

In prokaryotes, differentiation can be relatively simple. Examples are the formation of endospores in bacilli and heterocysts in cyanobacteria.

However, some bacteria exist as real organised populations like the aerial mycelia in streptomycetes and the fruiting body in myxobacteria. The developmental processes in these bacteria are more complicated and involve tactic and developmental cell movement, the exchange of chemical signals between cells, the coordinated construction of multicellular structures, and direct cell-cell interaction.

Myxobacteria are mainly characterised by their biphasic life cycle. Under normal growth conditions, myxobacteria grow and divide by transverse fission. When nutrients are depleted, cells migrate on an insoluble surface into aggregation centres from which the three dimensional structure, fruiting bodies, arise. Within the mature fruiting body, vegetative cells convert into myxospores. In addition, myxospores can be induced uncoupled from the developmental cell cycle by different chemical compounds.

Obviously, myxobacteria show properties of both unicellular and multicellular organisms and may lie on the boundary between the two kinds of organisms. Thus, they offer a good model system to investigate multicellular morphorgenesis and differentiation.

1.1. Myxobacteria

Myxobacteria are Gram-negative, rod-shaped soil bacteria that live on insoluble organic matter. They belong to the order *Myxobacterales* that includes 12 genera and about 40 species (Reichenbach, 1993; Reichenbach and Dworkin, 1992). The genome size of myxobacteria is rather large as compared to other bacteria. It ranges from 9.2 Mbp to 10 Mbp and has a G+C content of 67-72% (Chen *et al*., 1990; Neumann *et al*., 1992). Phylogenetically, myxobacteria belong to the δ subdivision of the proteobacteria, and are relatives of the genera *Bdellovibrio* and *Desulfovibrio* (Shimkets and Woese, 1992).

In the past years, two research model systems have been developed in myxobacteria: *Myxococcus xanthus* and *Stigmatella aurantiaca*. The fruiting body of *M. xanthus* is a simple mound, whereas *S. aurantiaca* forms a much more complicated fruiting body with a stalk and multiple sporangioles housing differentiated myxospores. But for *S. aurantiaca*, less bacterial genetic methods are available for the manipulation (Schairer, 1993).

1.1.1. Gliding motility

The motility by gliding over a solid surface plays an important role in the developmental cycle of myxobacteria (Reichenbach, 1984). In the case of *M. xanthus*, it has been demonstrated that the gliding motility involves two distinct systems: adventurous-motility system (A-motility) and social-motility system (S-motility). A-motility allows individual cells to move away from a colony edge, while S-motility allows cells to move as groups (Hodgkin and Kaiser, 1979). More than 37 genes are involved in the A-motility, while the S-motility needs the type IV pili, fibrils, and the lipopolysaccharide O-antigen of the cell surface (Bowden and Kaplan, 1998; Youderian, 1998).

In addition to A- and S-motility gene classes, at least three other gene classes have been demonstrated to be involved in the motility of *M. xanthus*: *mgl* (mutual gliding), *frz* (frizzy), and *dsp* (dispersed).

Mutations in *mgl* abolish both adventurous and social motility. Two genes, *mglA* and *mglB*, were found in the *mgl* locus (Stephens *et al*., 1989). The deduced amino acid sequence of the *mglA* product shows homology to small G-proteins of the eukaryotic *ras* family, suggesting a possible function of MglA in signal transduction (Hartzell and Kaiser, 1991a; Hartzell, 1997). The predicted amino acid sequence of the *mglB* gene product shows some similarity to one of the calcium-binding sites of yeast calmodulin (Hartzell and Kaiser, 1991b). The function of MglB is unclear.

The frequency of reversal of gliding direction is controlled by the *frizzy* system. The *frz* genes are homologous to the enteric bacterial chemotaxis genes for swimming although *M. xanthus* has no flagellum (Shi *et al*., 1993; Ward and Zusman, 1997). The role of the gene products of *frz*A, *frz*CD, *frz*E, *frz*F, *frz*G, and *frz*Z are predicated to be similar to those of their homologous proteins CheW, Tar (a methyl-accepting chemotaxis protein), CheA-CheY, CheR, CheB, CheY, respectively. In contrast to other methylated chemotaxis proteins that are membrane integral, FrzCD is a soluble cytoplasmic protein. FrzE is a hybrid protein contains sequences homologous to both CheA and CheY. It acts as both a kinase and a phosphatase. FrzB has no homologous protein in the enteric Che system. Its funtion is unknown.

Mutations in the *dsp* gene result in the loss of fibrils from cell surface as well as the loss of cohesion, group motility, and developmental ability (Arnold and Shimkets, 1988; Chang and Dworkin, 1996). This suggests that fibrils play an essential role in the social behaviour of myxobacteria.

1.1.2. Fruiting body formation

The life cycle of myxobacteria consists of a vegetative growth cycle and a developmental cycle (Fig. 1.1). The developmental cycle of myxobacteria, including fruiting body formation and sporulation, are triggered by the nutritional and physical changes of environment. During fruiting body formation, myxobacteria sense the depletion of nutrient, move rhythmically (rippling), aggregate together on a solid surface, construct multicellullar fruiting bodies (ca. 10^5 cells/fruiting body) and convert the vegetative cells into stress resistant myxospores inside the fruiting body (Dworkin, 1985). Additionally, sporulation can be induced independently from starvation by different chemical agents such as glycerol, DMSO, indole and its derivatives (Dworkin, 1994; Gerth *et al*., 1994; O'Connor and Zusman, 1997).

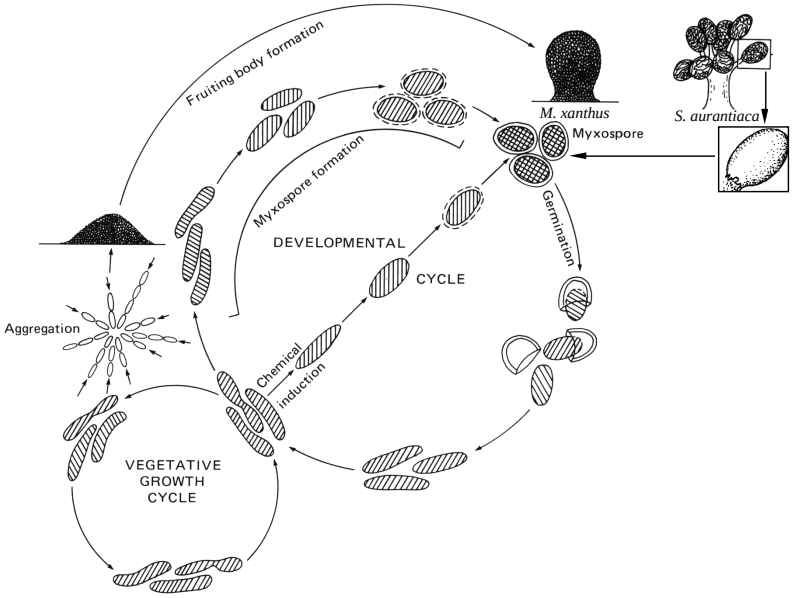


Fig. 1.1. Diagram of the life cycle of myxobacteria (Dworkin, 1985). The fruiting body of *M. xanthus*, in which myxospores are embedded in the slime mound, and the fruiting body of *S. aurantiaca*, which consists of a stalk and sporangioles, are illustrated.

Development of myxobacteria fruiting body requires intercellular communication. So far, at least five extracellular signals (Asg, Bsg, Csg, Dsg, and Esg) and a general starvation signal, (p)ppGpp, have been detected in *M. xanthus* (Downard *et al*., 1993; Dworkin, 1996; Hagen *et al*., 1978; Harris *et al*., 1998; Kuspa *et al*., 1992a; Kuspa *et al*., 1992b; LaRossa *et al*., 1983; Plamann *et al*., 1992). Cells with a mutation in a gene required for the production

of any of these signals are arrested in development at a specific stage. The development of such mutants can be rescued transiently by co-development with wild-type cells or with mutant cells of a different signaling group.

Since myxobacteria use mainly amino acids as a metabolic starting point to generate energy and synthesise other macromolecules, they employ a mechanism that senses the internal concentration of amino acids. It has been proven that the increased level of (p)ppGpp, in response to the amino acid limitation or heterologous expression of *E. coli relA* gene, will induce early developmental genes and lead to fruiting body formation in *M. xanthus*. This suggests (p)ppGpp to be one of the earliest starvation signals of development (Manoil and Kaiser, 1980a; Manoil and Kaiser, 1980b; Singer and Kaiser, 1995). Analysis of *M. xanthus relA* mutants showed that the intercellular production of (p)ppGpp is both necessary and sufficient to activate the developmental program, including the extracellular production of A-signal (Asg) (Harris *et al*., 1998).

B-signal (Bsg) functions in a very early stage of development. Only one gene, *bsgA*, has been found in the *bsg* locus, which encodes a 90.4-kDa intracellular ATP-dependent cytoplasmic protease (Gill and Cull, 1986; Gill *et al*., 1993). *bsg* Mutants fail to aggregate, fruit, sporulate, and are unable to express developmentally regulated genes.

A-signal is a mixture of amino acids and peptides that are generated by proteases. Mutation analysis has determined three gene loci for Asg, which are *asgA*, *asgB*, and *asgC*. They act at the early stage of development during pre-aggregation (Kuspa and Kaiser, 1989). The deduced amino acid sequence of *asgA* gene product contains two domains, one is homologous to the transmitter domain of histidine protein kinases and the other is homologous to the receiver domain of response regulators (Plamann *et al*., 1995). *asgB* encodes a putative DNA-binding protein that has a helix-turn-helix motif near the C-terminus. It appears to be a transcription factor that binds to the -35 region of the promoter (Plamann *et al*., 1994). *asgC* encodes the major sigma factor (*sigA*) in *M. xanthus* (Shimkets, 1999).

D-signal (Dsg) acts about 1 to 2 hours after the beginning of development. A single gene, *dsg*, has been determined in the *dsg* locus that encodes a protein with 50% sequence identity to the translation initiation factor IF3 of *E. coli*. Inactivation of *dsg* impairs partially development, i.e., aggregation is abnormal and delayed, and sporulation is reduced. It has been proven that the Dsg protein does act like IF3 in *M. xanthus*, suggesting that Dsg is involved in translation regulation during development (Cheng and Kaiser, 1989a; Cheng and Kaiser, 1989b; Cheng *et al*., 1994; Kalman *et al*., 1994; Rosenbluh and Rosenberg, 1989).

E-signal (Esg) is active at 3 to 5 hours after the beginning of development, and acts after Asg. Sequence and biochemical data indicated the *esg* genes to encode the E1α and E1β subunits of an α-keto acid dehydrogenase that is involved in amino acid and fatty acid metabolism (Downard and Toal, 1995; Toal *et al*., 1995).

C-signal (Csg) acts from about 6 hours after the beginning of development throughout the whole development cycle. One gene, *csgA*, is found in the *csg* locus. The *csgA* mutant is unable to ripple, aggregate, or sporulate (Shimkets and Asher, 1988). CsgA is an extracellular protein. It is associated with the cell surface (Shimkets and Rafiee, 1990). Sequence analysis revealed that CsgA shares homology with the family of short chain alcohol dehydrogenases. This suggests that CsgA may be an enzyme capable of monitoring the metabolic state of the cell during aggregation (Hartzell and Youderian, 1995; Lee and Shimkets, 1994). In addition, it has been proven that different developmental stages such as rippling, aggregation, and sporulation require different expression levels of CsgA, indicating that CsgA may act as an extracellular timer of development (Dworkin, 1996; Li *et al*., 1992).

Fruiting body formation of *S. aurantiaca* is stimulated by incandescent light (Qualls *et al*., 1978). Aggregation and differentiation of *S. aurantiaca* absolutely depend on the function of a pheromone, which is secreted by cells to initiate fruiting body formation. This pheromone designated stigmolone is a hydroxy ketone: 2,5,8-trimethyl-8-hydroxy-nonan-4–one, with a molecular mass of 200 (Hull *et al*., 1998; Plaga *et al*., 1998; Stephens *et al*., 1982).

The *mgl* genes and the *csgA* gene of *S. aurantiaca* have been cloned. The nucleotide sequences of *mglA* and *mglB* show 90% identity to thoses of *M. xanthus* corrsponding genes, while the *csgA* gene in *S. aurantiaca* has only 70% nucleotide sequence identity to the *csgA* of *M. xanthus* (Schairer, 1993). In addition, two other genes are known to be involved in fruiting body formation of *S. aurantiaca*, *fbfA* and *fbfB* (Silakowski *et al*., 1998; Silakowski *et al*., 1996). The deduced protein sequence of *fbfA* has 30% sequence identity to NodC of rhizobia, an N-acetylglucosamine-transferase. FbfB shows a significant homology to the galactose oxidase of *Dactylium dendroides*.

1.1.3. Transcriptional regulation in myxobacteria

During vegetative growth and development of myxobacteria, many genes are temporarily expressed at specific stages (Kroos and Kaiser, 1987; Kroos *et al*., 1986). In prokaryotes, gene regulation occurs mainly at the transcriptional level. A number of sigma factors have been shown to play an important role in gene regulation during myxobacterial life cycle. Six

sigma factors in *M. xanthus* have been cloned and identified. SigA is the house keeping sigma factor that shows high homology to σ^{70} of *E. coli* and σ^{43} of *Bacillus subtilis* (Biran and Kroos, 1997; Inouye, 1990). SigB and SigC are development-specific sigma factors (Apelian and Inouye, 1990; Apelian and Inouye, 1993). *sigC* is expressed in the very early stage of development. Deletion of *sigC* results in a distorted fruiting body. *sigC* mutants can initiate development in the presence of high levels of nutrient suggesting that SigC functions to prevent development without nutrient depletion. SigB is active in the late stage of differentiation. A *sigB* mutant undergoes normal fruiting body formation but produces spores without the spore-specific protein S1 (see 1.1.4). SigD is essential for both stationary phase and for multicellular differentiation (Ueki and Inouye, 1998). A deletion mutant of *sigD* exhibited growth defects during the late log phase and the stationary phase, with dramatically reduced cell viability. σ^{54} in *M. xanthus* has been cloned using a heterologous *rpoN* as a probe (Keseler and Kaiser, 1997). Any attempt to construct a *rpoN* null mutant failed, suggesting σ^{54} to be a vital protein for *M. xanthus*. Recently, an ECF (extracytoplasmic-function) sigma factor, encoded by *rpoE1*, has been identified in *M. xanthus* using a yeast two-hybrid system (Ward *et al*., 1998). An insertion into *rpoE1* affects the swarming of cells on nutrient-rich agar and the developmental aggregation during starvation. The function of the ECF sigma factor is proposed to play a role in the transcriptional regulation of genes involved in the motility behaviour during the whole life cycle.

In addition to sigma factors, an unique transcription factor, FruA, has been reported to be essential for fruiting body formation and sporulation in *M. xanthus* (Ogawa *et al*., 1996).

In *S. surantiaca*, the house keeping sigma factor (SigA) has been cloned and identified (Skladny *et al*., 1994). Moreover, two other sigma factors have been found to correlate with development: the expression of *sigB* is detected from the very beginning of fruiting body formation to the sporulation stage, while *sigC* appears late in the development, from stalk formation to sporulation stages. The genes regulated by the two sigma factors are unknown (Coudart, 1998).

1.1.4. HspA, a spore-specific polypeptide in *S. aurantiaca*, is a member of the small heat shock protein family

From indole induced spores of *S. aurantiaca* DW4/3-1, a polypeptide, HspA (originally named SP21), was isolated. It sediments with the spore membranes. Using specific antisera, HspA was detected in fruiting body derived spores, in heat shocked cells, and in oxygen

deprived cells, but not in vegetative cells growing under unstressed conditions (Heidelbach *et al*., 1993b).

Immunoeletron microscopy revealed HspA to be at the cell periphery in heat shocked cells and either at the cell periphery or within the cytoplasm in indole induced cells, often forming clusters. In fruiting body-derived spores, HspA was located mainly on the cell wall, preferentially at the outer periphery. Furthermore, HspA was found also to be associated with cellular remnants within the stalk and within the peripheral horizon next to the fruiting body (Lünsdorf *et al*., 1995).

The corresponding gene of HspA (*hspA*) was isolated from a λgt11 expression library using antisera against HspA. Its deduced amino acid sequence is homologous to other small heat shock proteins of plants. This suggests that HspA is a member of the small heat shock protein family (Heidelbach *et al*., 1993a).

Also in *M. xanthus*, several spore-specific proteins have been identified. Protein S and S1 are encoded by *tps* and *ops*, respectively (Inouye *et al*., 1983a; Inouye *et al*., 1983b). Protein S is a 19-kDa major protein component of the thick spore coat and synthesised at an early stage of development, while protein S1 is synthesised late in the development. Protein S1 accumulates inside the spores (Inouye *et al*., 1979a; Teintze *et al*., 1985). Protein S has two domains that are similar to the Ca^{2+}-binding domain of calmodulin and share sequence homology to β- and γ-crystallin of bovine lens. Protein U is synthesised during the late stage of development. It is secreted using a signal peptide and assembled on the surface of myxospores (Gollop *et al*., 1991; Inouye *et al*., 1979b). Protein C is a 30-kDa spore coat protein and synthesised earlier than protein S (McCleary *et al*., 1991).

Recently, a new spore-specific protein W has been found during the germination process of *M. xanthus* that is detected in the spore shell fraction and has a molecular mass of 41.5 kDa (Otani *et al*., 1998). It is proposed that protein W is synthesised at a very late stage in development.

1.2. Small heat shock proteins

As a universal protective mechanism, all organisms undergo a rapid molecular response to adapt to harmful environmental conditions, such as temperature fluctuation, starvation, and other physiological stresses. During these events, a subset of heat shock proteins (HSPs) or stress proteins is synthesised. Based on their sizes they are grouped in the following families: HSP100, HSP90, HSP70, HSP60, HSP40, HSP10 or sHSP (small heat shock protein), etc. Many HSPs act as molecular chaperones and have multiple cellular functions including the

folding and translocation of newly synthesised proteins, the refolding or degradation of confomationally damaged proteins, and the control of biological activity of specific regulatory proteins under normal growth as well as stress related conditions (review see (Bukau, 1999; Hartl, 1996; Morimoto *et al*., 1994).

The small heat shock protein family is composed of abundant and widely distributed stress proteins that have been found in Archaea, Bacteria and Eukarya. Although the members of this family have only limited sequence similarity (Caspers *et al*., 1995; Parsell and Lindquist, 1993), they are allocated to one family due to the following common features : (1). The molecular masses of the monomers range in size from 12 to 43 kDa (de Jong *et al*., 1998; de Jong *et al*., 1988; de Jong *et al*., 1993; Morimoto *et al*., 1994); (2). Their syntheses are induced by heat shock and other stressors (Heidelbach *et al*., 1993a; Klemenz *et al*., 1993; Landry *et al*., 1991); (3). There is a evolutionarily conserved region, homologous to the "α–crystallin domain" of the vertebrate eye lens α-crystallin protein, in the C-terminal half of these proteins (de Jong *et al*., 1998; Jakob and Buchner, 1994; Plesofsky Vig *et al*., 1992; Waters, 1995); (4). They tend to form large oligomeric complexes of 8 to 40 subunits with a molecular mass ranging from 125 kDa to 2 MDa (Arrigo *et al*., 1988; Chang *et al*., 1996; Ehrnsperger *et al*., 1997; Groenen *et al*., 1994; Liang *et al*., 1997).

Some sHSPs are constitutively expressed under physiological conditions. Examples are the sHSPs from yeast, *Drosophila*, *Xenopus*, and mammals (Gernold *et al*., 1993; Heikkila *et al*., 1991; Kurtz *et al*., 1986; Pauli *et al*., 1990). Their concentration depends on the cell type and organism, as well as on growth condition, on development and differentiation of the cell, etc (Bond and Schlesinger, 1987; Ciocca *et al*., 1993; Klemenz *et al*., 1993). During heat shock, typically, the concentration of small heat shock proteins in the cell increases 10 to 20 folds. This suggests sHSPs to have a function in thermotolerance.

1.2.1. Cellular localisation of small heat shock proteins

The localisation of sHSPs in the cell is variable. In plants, there are at least five classes of sHSP. Class I and II are found in the cytosol, the other three families in the endoplasmic reticulum, chloroplasts, and mitochondria, respectively (Waters, 1995). Dm-Hsp22 is one of the four sHSPs of *Drosophila* (Dm-Hsp22, 23, 26, and 27). It is located in the mitochrodria (Michaud *et al*., 1997). All other sHSPs from eukaryotes, including the five members in mammals (αA/αB-crystallin, Hsp25/27, HspL27, and p20), are found in the cytosol (Fink and Goto, 1997; Hickey *et al*., 1986; Kato *et al*., 1994; Lam *et al*., 1996). During stress induction, some sHSPs are redistributed and enriched inside the nucleus or in the periphery of the

nucleus (Arrigo and Welch, 1987; Collier and Schlesinger, 1986; Lavoie *et al*., 1993; Rossi and Lindquist, 1989).

sHSPs of prokaryote have been identified in the following bacteria: *Bacillus subtilis* (Henriques *et al*., 1997), *Bradyrhizobium japonicum* (Narberhaus *et al*., 1996), *Clostridium acetobutylicum* (Sauer and Durre, 1993), *Escherichia coli* (Allen *et al*., 1992), *Leuonostoc oenos* (Jobin *et al*., 1997), *Mycobacterium leprae* (Nerland *et al*., 1988), *Mycobacterium tuberculosis* (Chang *et al*., 1996), *Stigmatella aurantiaca* (Heidelbach *et al*., 1993b), *Streptomyces albus* (Servant and Mazodier, 1995), *Synechococcus vulcanus* (Roy and Nakamoto, 1998) and Archaeon *Methanococcus jannaschii* (Kim *et al*., 1998c). IbpA/IbpB from *E. coli* were found in the cytosol associated with inclusion bodies, while Hsp18 from *Leuonostoc oenos* was found to be associated with the cell membrane. The 16-kDa antigen from *Mycobacterium tuberculosis* is proposed to be on the outside of the cell wall. HspA is associated with the outer coat of the cell in *S. aurantiaca*.

1.2.2. Structural characteristics of small heat shock proteins

Most members of the sHSP family harbour a homologous sequence of about 80-100 residues in the C-terminal half of the protein, the so-called α-crystallin domain. Exceptions are yeast Hsp12 and Hsp9 (Jang *et al*., 1996; Orlandi *et al*., 1996). Phylogeny reconstruction indicates that multiple sHSPs were already present in the last common ancestor of pro- and eukaryotes. During the evolution of eukaryotes, animal and non-animal sHSPs developed from different ancestral gene copies (de Jong *et al*., 1998). No homology has been observed in the C-terminal extension that is downstream of the α-crystallin domain. The size and the sequence of the N-terminal region of sHSPs are highly variable (de Jong *et al*., 1998; Merck *et al*., 1993b).

The secondary structures of α-crystallin and sHSPs have been elucidated by far-UV circular dichroism spectroscopy and Fourier-transform infrared analyses. These sHSPs contain predominantly β-sheets (40-50%), few α-helix conformation (10-20%), and random turns and coils (Farnsworth *et al*., 1998; Farnsworth *et al*., 1997; Merck *et al*., 1993a; Surewicz and Olesen, 1995). In the case of α-crystallin, increased temperature and concentration result in increased α-helical part with a compensatory decrease in β-sheets (Farnsworth *et al*., 1997).

The tertiary structure of sHSP is speculated to be determined by two domains: the hydrophilic C-terminal domain and the hydrophobic N-terminal domain (Groth Vasselli *et al*.,

1995). In agreement with this model, the isolated C-terminal domains of α-crystallins form soluble dimers and tetramers, while the N-terminal domains form insoluble large aggregates.

It is a remarkable feature of the sHSP family that most members tend to assemble into a large oligomeric complex. The multimerisation of sHSP seems to be necessary for the interaction of sHSP with unfolded proteins, to act as a chaperone. Deletion of the N-terminus of αA-, αB-crystallin, and Hsp25 resulted in the loss of the capacity to oligomerise correctly and to suppress thermally induced protein aggregation (Merck *et al*., 1993b). To support this speculation, a member of a novel class of this family found in *Caenorhabditis elegans*, a 12.6–kDa sHSP that is exceptionally a monomer, does not function as a molecular chaperone *in vitro* (Leroux *et al*., 1997a).

Since sHSPs from different organisms form different oligomers, the quaternary structure of sHSPs complexes should be also variable (Groenen *et al*., 1994; Haley *et al*., 1998). Among the several proposed models for sHSP quaternary structures, the micellar model seems to accommodate best the variability in subunit stoichiometry and particle morphology in many sHSPs (Farnsworth *et al*., 1998; Leroux *et al*., 1997b). In this model the elongated sHSP subunits are arranged in such a way that the hydrophilic C-terminal domain of each subunit is on the surface of the oligomeric complex and keeps the complex soluble. The hydrophobic N-terminal domain is inside the complex, and provides the driving force for subunit aggregation. A central cavity may exist in the sHSP complex that accommodates the N-terminal domains of varying lengths and sequence. The flexibility of the C-terminal domains permits sHSPs to address the requirements for binding various proteins and small ligands.

The first sHSP crystal structure was determined with HSP16.5 from *Methanococcus jannaschii* (MjHSP16.5). The monomeric folding unit of MjHSP16.5 is a composite β–sandwich in which one of the β-strands comes from a neighbouring molecule. Twenty-four monomers form a complex which is hollow, spherical, and octahedral symmetric, with eight trigonal and six square ‘windows’. The sphere has an outer diameter of 120 Å and an inner diameter of 65 Å. However, the N-terminal 32 amino acid residues of the protein were higly disordered in this complex. Their structure and role remains a matter of speculation (Kim *et al*., 1998a; Kim *et al*., 1998b).

1.2.3. Function and chaperone properties of small heat shock proteins

sHSPs are suggested to be involved in various processes, including RNA stabilisation, protease inhibition, actin microfilament assembly, cellular growth, transcription, and

differentiation (Morimoto *et al*., 1994). It has been reported that over-expression of sHSP encoding genes increased heat resistance of the cell (Allen *et al*., 1992; Aoyama *et al*., 1993; Knauf *et al*., 1992; Mehlen *et al*., 1993; Rollet *et al*., 1992; van den *et al*., 1994; Yeh *et al*., 1997). Furthermore, expression of Hsp27/25 blocked apoptosis, inhibited the activation of the transcription factor NF-κB, increased the stabilisation of microfilaments, and protected cells against oxidative stress and the action of the tumour necrosis factor (TNFα) (Arata *et al*., 1997; Lavoie *et al*., 1993; Mehlen *et al*., 1997a; Mehlen *et al*., 1997b; Punyiczki and Fesus, 1998; Welsh and Gaestel, 1998). However, sHSPs from different organisms seem to have different biological activities. Mutants in sHSP from *Dictyostelium* were unable to develop thermotolerance (Loomis and Wheeler, 1982), while the inactivation of the gene that encodes Hsp26 of yeast did not cause any detectable phenotypic effect (Petko and Lindquist, 1986).

Increasing numbers of *in vitro* assays demonstrated that sHSPs function as molecular chaperones by selectively binding to denatured proteins, preventing thermal aggregation of proteins, and facilitating protein folding or refolding processes (Chang *et al*., 1996; Collada *et al*., 1997; Horwitz, 1992; Jakob *et al*., 1993; Lee *et al*., 1995). They show little or no substrate specificity and have a high affinity for unfolded, but not for aggregated or native polypeptides. Interestingly, many sHSPs function in an ATP-independent way. However, αB crystallin has been proven recently to be an ATP-enhanced molecular chaperone (Muchowski and Clark, 1998). Chaperone function of sHSP seems to be incomplete. sHSPs are unable to promote the correct refolding of the sHSP-stabilised proteins. Therefore, it is supposed that sHSPs may function in co-operation with other chaperones in the refolding process. The first evidence for this assumption is that the refolding of heat-denatured citrate synthase bound to mammalian Hsp25 can be reactivated by Hsp70 (Ehrnsperger *et al*., 1997). In support of this model, it has been observed that the Hsp18.1 (an sHSP from plant) bound heat-denatured firefly luciferase can be reactivated in the presence of rabbit reticulocyte or wheat germ extracts in an ATP-dependent process (Lee *et al*., 1997). Recently, it was shown that IbpB from *E. coli* functions cooperative with a multichaperone network including GroEL/GroES and DnaK/DnaJ/GrpE (Veinger *et al*., 1998). Taken together, sHSPs are classified to be the "junior" members of the chaperone family (Jakob and Buchner, 1994).

1.3. Transcriptional regulation of heat shock genes

The heat shock response is mainly regulated at the transcriptional level (Mager and De Kruijff, 1995). Despite of structural and functional conservation of HSPs during evolution, the transcriptional regulation of heat shock genes is quite diverse.

1.3.1. Transcriptional regulation of heat shock genes in eukaryotes

In eukaryotes, activation of heat shock genes is mainly mediated by binding of transcription factors (HSFs) to a highly conserved, *cis*-acting DNA sequence, termed heat shock element (HSE) (Wu, 1995). All HSEs are composed of multiple repeated 5-bp sequences (5'-nGAAn-3'). These repeats are continuous but arranged in alternating orientation, i.e., 5'-nGAAnnTTCnnGAAn-3' (Amin *et al*., 1988; Xiao and Lis, 1988). The number of the 5-bp units in a functional HSEs can vary. At least three units are required for the heat dependent transcription, some HSEs harbour eight continuous repeats (Fernandes *et al*., 1994; Morimoto *et al*., 1994).

The activity of HSF is regulated by stress induction at the level of both DNA binding and transcriptional activation. Binding of HSF depends on its oligomerisation, whereas transcriptional activation correlates with its heat shock induced phosphorylation (Lis and Wu, 1992). So far, HSFs have been isolated from human, mouse, chicken, *Drosophila*, tomato, and yeast (Jakobsen and Pelham, 1991; Nakai and Morimoto, 1993; Nakai *et al*., 1997; Scharf *et al*., 1993; Schuetz *et al*., 1991). These analyses revealed a multiple gene family of HSF. Within the same species, members of the HSF family show approximately 40% amino acid sequence identity, mainly within the DNA binding and oligomerisation domains. Comparison of HSF1 from human, mouse and chicken revealed a high sequence similarity (85-95% amino acid identity). This suggests a common ancestral progenitor of the HSFs (Morimoto, 1993; Nakai and Morimoto, 1993).

HSFs, as many transcriptional regulators, are constitutively expressed in enkaryotic cells and stored in a latent form under normal growth conditions. The existence of multiple HSFs suggests that they may respond to diverse developmental and environmental changes. Eventually, it has been proven that the vertebrate HSF1 and HSF2 have different roles in the transcriptional regulation of heat shock genes. HSF1 is activated to correspond to the general stress inducers whereas HSF2 is induced during development and differentiation (Baler *et al*., 1993; Murphy *et al*., 1994; Sarge *et al*., 1993; Sistonen *et al*., 1992). In the unstressed cells, HSF1 is maintained as a non-DNA-binding monomer in the cytoplasm. In response to heat shock, HSF1 is translocated into the nucleus, where it forms trimers. The HSF trimers then trigger the HSE dependent transcription. Activation of HSF2 is linked to proteolysis, and to the activation of the ubiquitin dependent proteasome. In response to the accumulation of non–native polyubiquitinated proteins, HSF2 is activated from the inert dimer to the DNA-binding trimer. This leads to the transcription of HSE regulated genes (Morimoto, 1999).

Chicken HSF3 is activated after severe heat shock stimulation. In contrast to chicken HSF1 that is activated immediately after heat shock, activation of chicken HSF3 is delayed and sustained for a long period (Nakai *et al.*, 1995; Tanabe *et al.*, 1997). Human HSF4 has no property of a transcriptional activator (Nakai *et al.*, 1997). The control of HSF binding to DNA is bypassed in the budding yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. The HSF is bound to HSEs as a trimer before and after heat shock (Gross *et al.*, 1990; Jakobsen and Pelham, 1988; Sorger and Nelson, 1989). Activation of the HSF in yeast correlates with increased phosphorylation at serine and threonine residues (Sorger, 1990). Furthermore, the unique HSF from yeast is distinguished from the HSFs of higher eukaryotes by having two transcriptional activation domains that bind to different HSEs in response to different stimuli (Santoro *et al.*, 1998).

In addition to the HSE-dependent transcription, some other transcription regulation motifs have been reported. Genes that are responsible for the protection of the endoplasmic reticulum (ER) are regulated *via* the transcription activator Hac1 that binds to the unfolded protein response element (UPRE), a 22 bp sequence (Cox and Walter, 1996). A stress response element (STRE) with the consensus sequence AGGGG or CCCCT is found in yeast. It is involved in general stress gene expression and is found in the promoter of some yeast heat shock genes (Ruis and Schuller, 1995).

1.3.2. Transcriptional regulation of heat shock genes in prokaryotes

Transcriptional regulation of heat shock genes in prokaryotes is achieved either by alternative sigma factors or by negative control systems.

Two major alternative sigma factors σ^{32} and σ^{E} (σ^{24}) are involved in the heat shock response of *E. coli*. σ^{32} is induced by stressors acting in the cytoplasm, while the σ^{E} is induced by extracytoplasmic stressors (review see (Connolly *et al.*, 1999; Yura *et al.*, 1993)). The transcription of σ^{32} (encoded by *rpoH* gene) occurs from four promoters (P1, P3, P4, and P5). P3 is recognised by RNA polymerase bearing σ^{E}, whereas the other three promoters are recognised by RNA polymerase containing σ^{70}. At 30°C, the P1 and P4 promoters account for more than 90% of *rpoH* transcription. At increasing temperature, transcription from P3 becomes more prominent, and at 50°C, P3 is the only active promoter. The activity of P5 is weak and totally dependent on the cAMP receptor protein (CRP).

σ^{32} dependent regulation of heat shock response has been found in other Gram-negative bacteria, especially in the α and γ subgroups of proteobacteria (Nakahigashi *et al.*, 1995).

In *B. subtilis* another alternative sigma factor, σ^B, is employed. The induction of about 40 heat shock genes that belong to Class II heat shock gene family of this bacterium are controlled by RNA polymerase containing σ^B. Class I heat shock genes of *B. subtilis* are negatively regulated *via* the interaction of HrcA repressor with an inverted repeat DNA sequence, designated CIRCE element in the promoter region of heat shock genes. The CIRCE dependent negative control mechanism has been found in many eubacteria (Avedissian and Lopes Gomes, 1996; Roberts *et al*., 1996; Segal and Ron, 1996a; Segal and Ron, 1996b). The regulation of Class III heat shock genes of *B. subtilis* are not well understood. They are expressed using a σ^{43} dependent promoter, but the regulation by different mechanisms remains to be elucidated (Hecker *et al*., 1996; Schulz *et al*., 1997).

Besides HrcA/CIRCE and alternative σ^{32} control systems (Babst *et al*., 1996; Narberhaus *et al*., 1996), an additional negative control system has been detected in *Bradyrhizobium japonicum* that depends on a DNA-element named ROSE (repression of heat shock gene expression). It is a conserved DNA element of about 100 bp that is located between the transcription and translation start sites of the first gene of each operon (Narberhaus *et al*., 1998; Narberhaus *et al*., 1997).

For *Streptomyces coelicolor*, it has been reported that the regulation of dnaK operon is governed by a negative control system. A repressor protein HspR interacts with the IR element in the promoter region of heat shock genes (Bucca *et al*., 1995; Bucca *et al*., 1997). In contrast, the two *groE* operons of this organism contain two CIRCE elements in their promoter region (Duchene *et al*., 1994a; Duchene *et al*., 1994b).

In the halophilic archeon *Haloferax volcanii*, a conserved archaeal consensus TATA motif has been found in the promoter of two heat responsive cct (chaperonin-containing Tcp-1) genes. This suggests a regulation system that is similar to that of eukaryotes (Kuo *et al*., 1997).

1.4. The aims of this work

It is mentioned above that *S. aurantiaca* is characterised by its biphasic life cycle. Thus, research on the differentiation-specific genes in this organism would reveal some clues to the molecular switch of the two different life cycles. *hspA* is the first heat shock gene identified in *S. aurantiaca*. Its expression is associated to both cell stress response and differentiation. Obviously, it is a good candidate in the aim to understand regulation of gene expression in *S. aurantiaca*. Furthermore, no gene has been studied at the transcriptional regulation level in this organism so far. The study on *hspA* expression would add novel knowledge to the

molecular genetics of this organism. The aim of this thesis is to elucidate the transcriptional regulation pattern of *hspA* as well as the function and the possible chaperone properties of the HspA protein. The following research work was performed:

- Characterisation of *hspA* locus in *S. aurantiaca*.
- Examination of *hspA* expression under different conditions, determination of its transcriptional initiation site, and analyses of its corresponding sigma factor(s).
- Establishment of an ectopic recombination system in *S. aurantiaca*.
- Delimitation of the *hspA* promoter region and determination of probable regulatory elements involved in *hspA* transcription.
- Construction of *hspA* deletion mutant and analysis of the HspA function *in vivo*.
- Biochemical characterisation of possible chaperone properties of HspA.

II. Results

Part I. Transcriptional regulation of the *hspA* gene

2.1. Molecular cloning and sequence analysis of a restriction fragment from *S. aurantiaca* DW4/3-1 that harbours *hspA* and its flanking regions

In the work of M. Heidelbach, a *Hpa*II fragment harbouring the *hspA* gene of *S. aurantiaca* wild-type strain DW4/3-1 was cloned into a λgt11 expression library. Subcloning of this fragment resulted in plasmid pL4 (Heidelbach *et al*., 1993a). When Southern hybridisation was performed with *Pst*I restricted or *Bam*HI restricted genomic DNA of *S. aurantiaca* DW4/3-1 using the insert of pL4 as a probe, two *Pst*I fragments (5.7 kbp and 4.7 kbp, respectively) and three *Bam*HI fragments (3.9 kbp, 1.9 kbp, and 1.5 kbp, respectively) have been detected (Inaugural dissertation of M. Heidelbach, 1992). However, sequence analysis showed that there is no *Pst*I site in the insert of pL4. Furthermore, there are two *Bam*HI sites in the *hspA* open reading frame (ORF) that are 36 bp apart from each other. Theoretically, only one *Pst*I fragment and two *Bam*HI fragments should be detected (the 36 bp fragment is too small to be detected) in this Southern analysis. For these unexpected results, there are two possible explanations: either there are two copies of the *hspA* gene in *S. aurantiaca* DW4/3-1 genome or a recombination occurred during the cloning process resulting in two unrelevant *Hpa*II fragments ligated together before they were cloned into λgt11. To characterise the *hspA* locus, to analyse the transcriptional regulation of *hspA*, and to construct a *hspA* deletion mutant, it was thus necessary to isolate a large restriction fragment that harbours the *hspA* gene and more than 1000 bp of its upstream and downstream regions from the genome of DW4/3-1.

2.1.1. Isolation of *hspA* gene from the genome of DW4/3-1

Genomic DNA of DW4/3-1 was digested with different restriction enzymes. Southern hybridisation was performed with a ^{32}P-labelled PCR product of the *hspA* gene (EMBL/GeneBank: M94510, bp 568-bp 1134), which contains the whole coding region of *hspA* determined by M. Heidelbach. The PCR product was synthesised by the primer pair A11 and A12 using pL4 as template.

Fig. 2.1 shows that only one fragment was detected in the Southern analysis of *Pst*I (ca. 5.5 kbp), *Xma*I (ca. 2.8 kbp), *Eag*I (ca. 2.5 kbp), and *Xho*I (ca. 9 kbp) restricted chromosomal DNA. This result indicates clearly that only one copy of the *hspA* gene is in the genome of *S. aurantiaca* DW4/3-1.

No clear signal was detected in *Eco*RI, *Hin*dIII, *Sal*I, *Cla*I, and *Kpn*I restricted genomic DNA. The possible reason is that the restriction fragments that carry the *hspA* gene are too large to be well separated in the agarose gel.

It seems that the 5.5 kbp *Pst*I fragment harbouring the *hspA* gene is suitable for further work, since it contains totally about 5 kbp flanking sequences of the *hspA* gene. It is very probable that this *Pst*I fragment harbours a 5'-flanking region of *hspA* that is long enough to contain its whole promoter region.

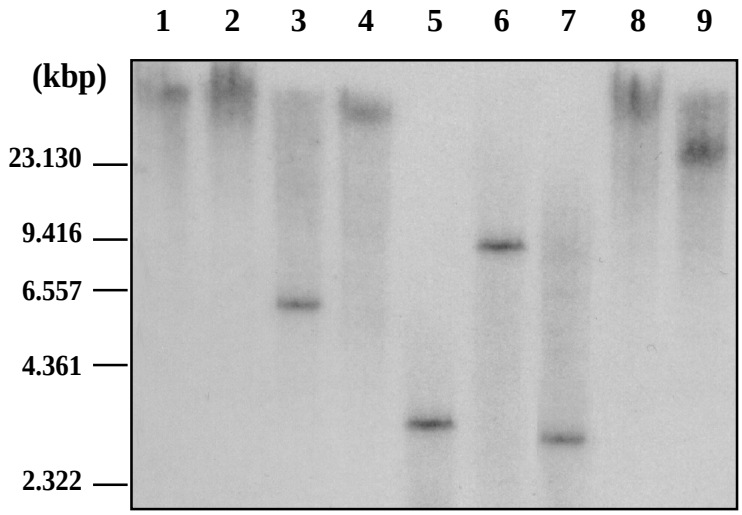


Fig. 2.1. Southern analysis of *S. aurantiaca* DW4/3-1 genomic DNA digests. In lanes 1-9, 10 μg DNA was digested with *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, *Xma*I, *Xho*I, *Eag*I, *Cla*I, and *Kpn*I, respectively and separated in a 1% agarose gel. Southern hybridisation was performed using the ^{32}P-labelled PCR product of the *hspA* gene (EMBL/GeneBank: M94510, bp 568-bp 1134) as a probe.

To establish a gene library that contains the *hspA* gene, genomic DNA of DW4/3-1 was digested with *Pst*I. The restriction fragments were separated by agarose electrophoresis. The fragments ranging from 4 to 6 kbp were recovered from agarose gel by electroelution and inserted into the *Pst*I site of the vector pBC SK+ (Stratagene). The recombinant plasmids were transferred into *E. coli* by electroporation. Transformants containing the *hspA* gene were detected by colony hybridisation using the same gene probe. Dot and Southern hybridisation analyses confirmed that pSH1 harboured the 5.5 kbp *Pst*I fragment containing the *hspA* gene from the genome of *S. aurantiaca* DW4/3-1(data not shown).

2.1.2. Determination of the *hspA* open reading frame

Sequencing of the pSH1 insert was started in the coding region of the *hspA* gene, and the sequence data were compared with those published (Heidelbach *et al*., 1993a). A difference was found between the two sequences. An additional G was found at bp +510 downstream of the translation start site of the *hspA* in this work. This shifted the stop codon of *hspA* 54 bp upstream of the published stop codon of *hspA*. The corrected *hspA* ORF is 510 bp long and encodes a polypeptide of 169 amino acids with the calculated molecular mass of 19357.66 Da. This protein was designated HspA instead of SP21. Moreover, a serine residue was found in HspA at the position 111 instead of a threonine residue at the same position of SP21.

A putative factor-independent termination structure was found downstream of the *hspA* (Fig. 2.2)

The deduced amino acid sequence of HspA was compared with other small heat shock proteins including the members of Class I and II small heat shock proteins of plants, and SP21 (Fig. 2.3). As pointed out in the Introduction, the conserved region of different small heat shock proteins lies mainly in the α-crystallin domain. This region is conserved in HspA. The overall sequence identity between HspA and small heat shock proteins from plants ranges from 25% to 31%.

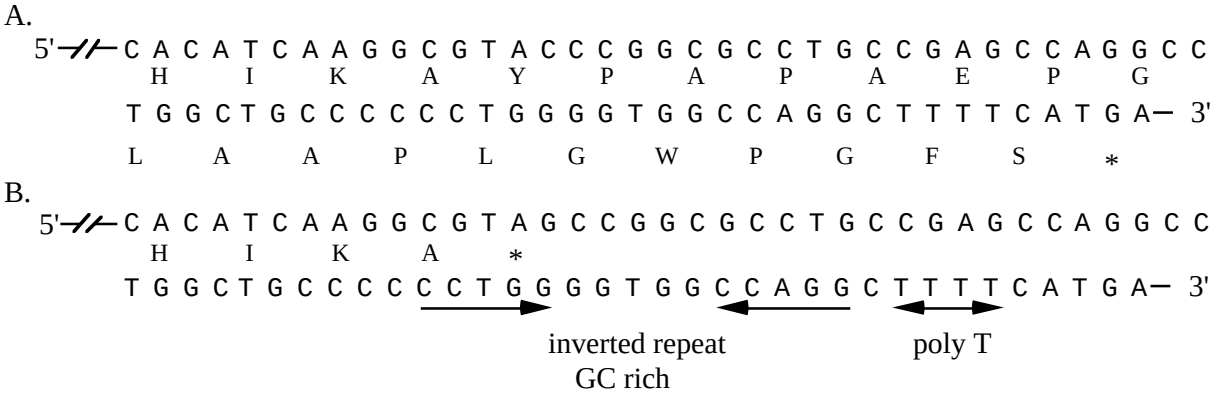


Fig. 2.2. Partial 3'-terminal sequence of the published *hspA* ORF (A) and the corrected *hspA* ORF (B). The factor-independent termination structure downstream of the corrected *hspA* ORF consists of two sequences: the inverted repeat is shown by arrows; the short poly T sequence (poly U in the mRNA) is shown by a double arrowhead.

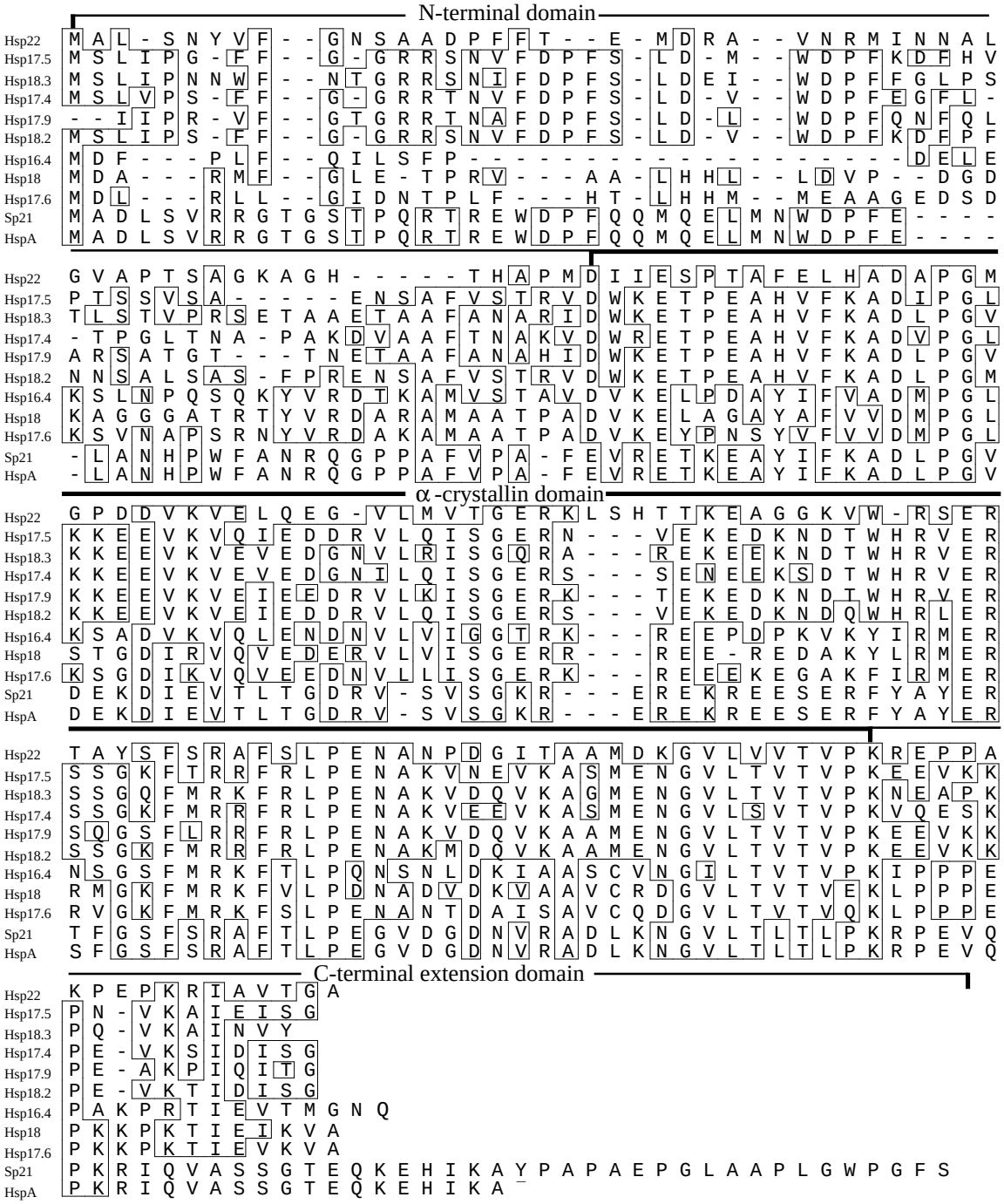


Fig. 2.3. Alignment of the amino acid sequence of HspA and other small heat shock proteins. The identical amino acids are boxed. The N-terminal domain, α-crystallin domain and C-terminal extension domain are lined over the sequences (Leroux *et al.*, 1997b). Hsp22: Chloroplast sHSP from *Chlamydomonas reinhardtii* (Grimm *et al.*, 1989); Hsp17.5: sHSP from *Glycine max* (Nagao *et al.*, 1985); Hsp18.3: Class I sHSP from *Chenopodium rubrum* (Knack *et al.*, 1992); Hsp17.4: Class I sHSP from *Arabidopsis thaliana* (Takahashi and Komeda, 1989); Hsp17.9: sHSP from *Pisum satiyum* (Lauzon *et al.*, 1990); Hsp18.2: Class I sHSP from *Medicago sativa* (Gyorgyey *et al.*, 1991); Hsp16.4: Class II sHSP from *Funaria hygrometrica* (Waters and E., 1999); Hsp18:

Class II sHSP from *Zea mays* (Atkinson *et al.*, 1993); Hsp17.6: Class II sHSP from *Lycopersicon esculentum* (Kadyrzhanova *et al.*, 1998); Sp21: sHSP from *S. aurantiaca* (Heidelbach *et al.*, 1993a); HspA: sHSP from *S. aurantiaca*, this work.

2.1.3. Sequence determination of the *Pst*I fragment downstream of the *hspA*-stop codon

It was mentioned above that the Southern analysis of *Pst*I restricted chromosomal DNA of DW4/3-1 using the insert of pL4 as a probe yielded an unexpected result. One possible reason is that the insert of pL4 was a result of a ligation of two unrelevant *Hpa*II fragments. One of the fragments contains the *hspA* and the other one originates from a genomic region of DW4/3-1 that does not flank the *hspA.* Southern analysis using the insert of pSH1 as a probe supported this assumption as it detected only one fragment in *Pst*I restricted chromosomal DNA of DW4/3-1. Further sequencing downstream of *hspA* confirmed this assumption. As shown in Fig. 2.4, the sequence downstream of bp 389 (relative to the *hspA*-stop codon) of pL4 insert is different from that of pSH1. A *Hpa*II restriction site is just located at the site where the pL4 and pSH1 inserts start to be different. It is obvious that the insert of pL4 is a hybrid fragment.

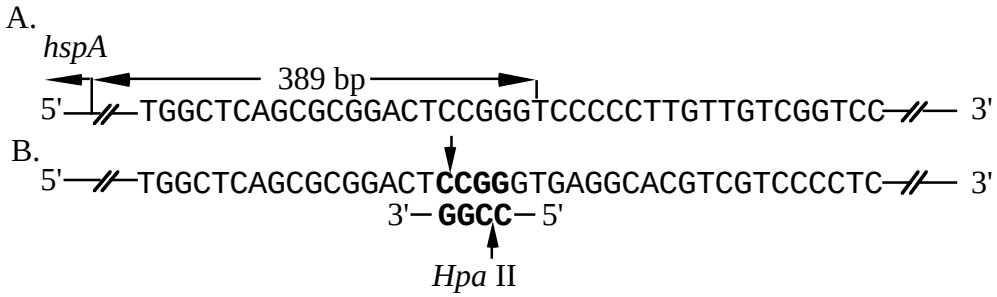


Fig. 2.4. The difference between the insert of pSH1 (A) and that of pL4 (B). The *Hpa*II site is indicated.

Moreover, a new ORF of 1323 bp was found in a divergent orientation to *hspA*. This ORF encodes a polypeptide of 440 amino acids. Its stop codon is localised 66 bp downstream of the *hspA*-stop codon. The start codon is ATG and the stop codon is TAA. A perfect putative ribosome-binding site was found 7 bp upstream of the start codon. The deduced amino acid sequence of this ORF was analysed through the National Centre for Biotechnology Information BLAST network server to search for homologous sequences. The result showed that this polypeptide has a significant homology (31% to 33%) to the microsomal epoxide hydrolases from mammals (Fig. 2.5).

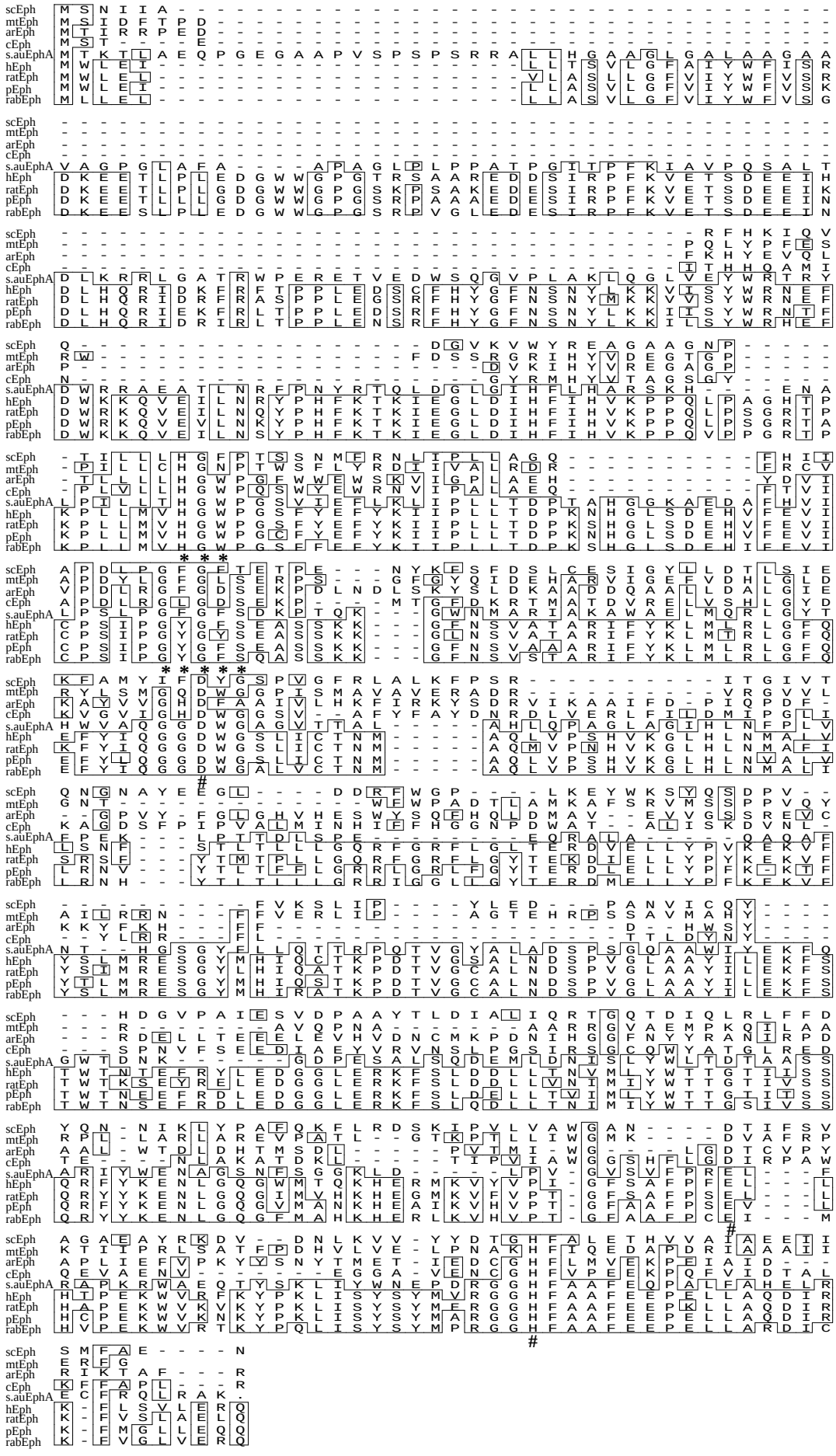

scEph
mtEph
arEph
cEph
s.auEphA
hEph
ratEph
pEph
rabEph

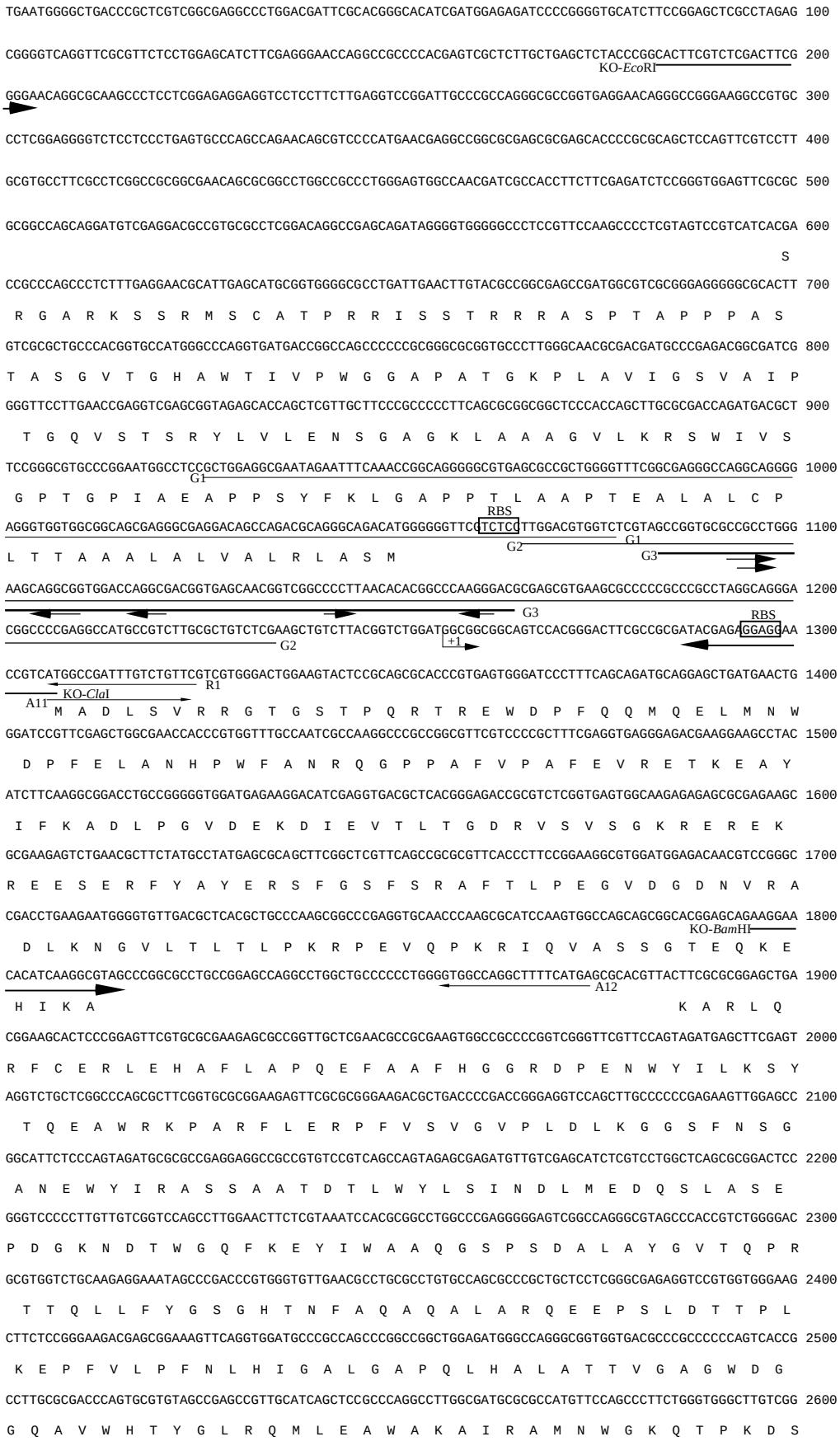
Fig. 2.5. Alignment of the amino acid sequence of EphA from *S. aurantiaca* and other epoxide hydrolases. The identical amino acids are boxed. The N-terminus conserved regions are marked by "*" below the sequence. The putative catalytic residues are marked by "#" below the sequence. scEph: epoxide hydrolase (EH) from *Saccharomyces cerevisiae* (SWISS-PROT: P53750); mtEph: EH from *Mycobacterium tuberculosis* (SWISS-PROT: Q50600); arEph: EH from *Agrobacterium radiobacter* (Rink *et al*., 1997); cEph: EH from *Corynebacterium* sp. (Misawa *et al*., 1998); s.auEH from *S. aurantiaca* DW4/3-1, this work; hEph: human microsomal EH from *Homo sapiens* (Hassett *et al*., 1994); ratEph: rat microsomal EH from *Rattus norveqicus* (Falany *et al*., 1987); pEph: pig EH from *Sus scrofa* (DDBJ/EMBL/GenBank: G1840391); rabEph: rabbit microsomal EH from *Oryctolagus cuniculus* (Hassett *et al*., 1989).

This result suggests that this ORF encodes an epoxide hydrolase. It is named *ephA* (epoxide hydrolase). The gene product is designated EphA. It is noteworthy that the sequence similarity between EphA from *S. aurantiaca* DW4/3-1 and the epoxide hydrolases from other bacteria and yeast is relatively low (12% to 15%).

The soluble and microsomal epoxide hydrolases of mammals belong to the family of α/β–hydrolase fold enzymes (Arand *et al*., 1994; Beetham *et al*., 1995; Lacourciere *et al*., 1993; Pries *et al*., 1994). The two N-terminal regions: HGX and GarGXS (X = any amino acid, ar = aromatic residue), which are conserved in α/β-hydrolases and epoxide hydrolases, are also found in EphA.

Three amino acid residues have been identified or proposed to form the catalytic centre of soluble and microsomal epoxide hydrolases, and of the epoxide hydrolase of an *A. radiobacter* strain AD1 (Arand *et al*., 1996; Beetham *et al*., 1995; Bell and Kasper, 1993; Rink *et al*., 1997). A nucleophilic amino acid (Nu) is conserved in the nucleophilic elbow sequence Sm-X-Nu-X-Sm-Sm (Sm = small residue) defined by Ollis *et al.* (Ollis *et al*., 1992), a histidine residue is highly conserved among the α/β-hydrolase fold enzymes that is located proximal to the C-terminus, and an acidic residue is located 26 to 34 residues upstream of the conserved histidine residue. Based on the sequence alignment, Asp237 (the putative nucleophilic residue), Glu389, and His416 are supposed to be the catalytic residues of the EphA of *S. aurantiaca* DW4/3-1.

A.



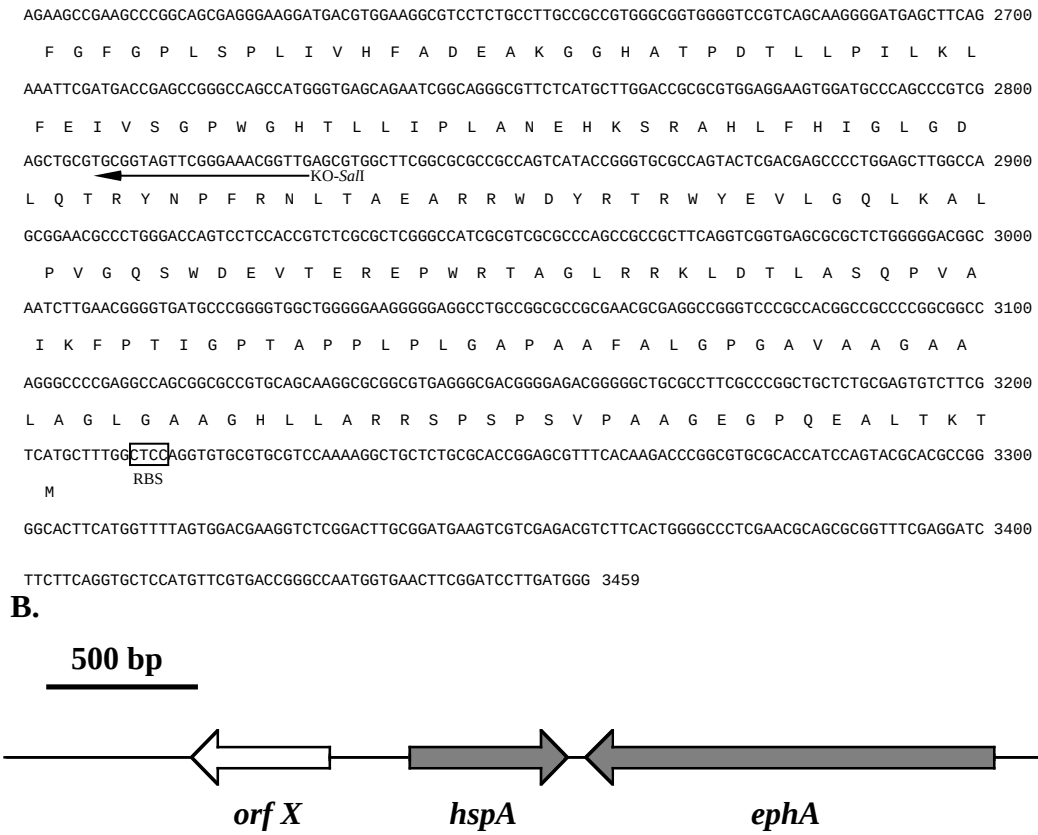


Fig. 2.6. A. Sequence of part of the pSH1 insert and the deduced amino acid sequence of *hspA*, *ephA*, and *orfx*. The putative ribosome-binding sites (RBS) are boxed. Some important regions are also indicated in this figure: "+1" shows the transcriptional start site of *hspA*; A11 and A12 show the primers used to generate *hspA* PCR product (2.1.1, 2.7.1); R1 is the primer used in primer extension assay (2.2.2); G1, G2 and G3 are the DNA fragments used in the gel retardation assays (2.4.2). The arrows on G3 indicate the inverted repeat regions on G3 (2.4.3.3). KO-*Eco*RI, KO-*Cla*I, KO-*Bam*HI, KO-*Sal*I are the primers used to construct the *hspA* deletion mutant (2.6.1.1). B. Schematic map of part of the pSH1 insert. *orfX* and *ephA* are the two open reading frames detected in the upstream and downstream regions of *hspA*, respectively.

2.1.4. Analysis of the upstream sequences of *hspA*

The 1.4 kbp upstream region of *hspA* has been sequenced. An ORF of 456 bp was found 256 bp upstream of the *hspA* that encodes a polypeptide of 151 amino acids. This ORF is arranged in a divergent orientation to *hspA*.

A putative ribosome-binding site was found 10 bp upstream of the start codon. However, no sequence has been found to be homologous to the deduced amino acid sequence of this

ORF when searching in the National Centre for Biotechnology Information BLAST network server. Therefore, it is named temporarily *orfX*.

Altogether, 3459 bp of the insert of pSH1 were determined (Fig. 2.6). Sequence data indicate that the *hspA* is located near the centre of this fragment. Thus, pSH1 contains sufficient up- and downstream flanking sequences of the *hspA* for further work.

2.2. Characterisation of *hspA* transcription

2.2.1. Analysis of the *hspA* transcript by Northern hybridisation

Sequence analysis of the pSH1 insert revealed no other ORF in the 5'- and 3'-flanking regions of *hspA* to be arranged in the same orientation as the *hspA*. This suggests *hspA* to be located in a monocistronic operon. This assumption was confirmed by the analysis of the *hspA* transcript that was induced either by heat shock or by indole treatment.

Northern hybridisation was performed with total RNA isolated from unstressed DW4/3-1 cells, from DW4/3-1 cells that were subjected to heat shock and from DW4/3-1 cells were treated with indole using ^{32}P-labelled *hspA* (see 2.1.1) as a probe.

The results (Fig. 2.7) revealed that the expression of *hspA* was induced dramatically under both stress conditions. Under heat shock conditions, the amount of *hspA* mRNA increased just a few minutes after the temperature up-shift, reached the maximum level after about 15 minutes, and decreased slightly thereafter. During indole treatment, the amount of *hspA* mRNA increased about 30 minutes after addition of indole and reached the maximum level after about 6 hours.

Interestingly, the *hspA* mRNA patterns were not identical under these two stress conditions. Two *hspA* transcripts were detected under heat shock conditions: one is about 650 nucleotides, and the other is about 700 nucleotides in length. But only the 650 nucleotide transcript was observed during indole treatment. It is not clear whether the 700 nts *hspA* mRNA is generated due to a second transcriptional initiation site of *hspA* or a transcription passing over the transcription terminator of *hspA*.

Considering the size of *hspA* gene (510 bp) and the size of *hspA* mRNA (650 to 700 nts), it is obvious that *hspA* is in a monocistronic operon. This supports the assumption from sequence analysis.

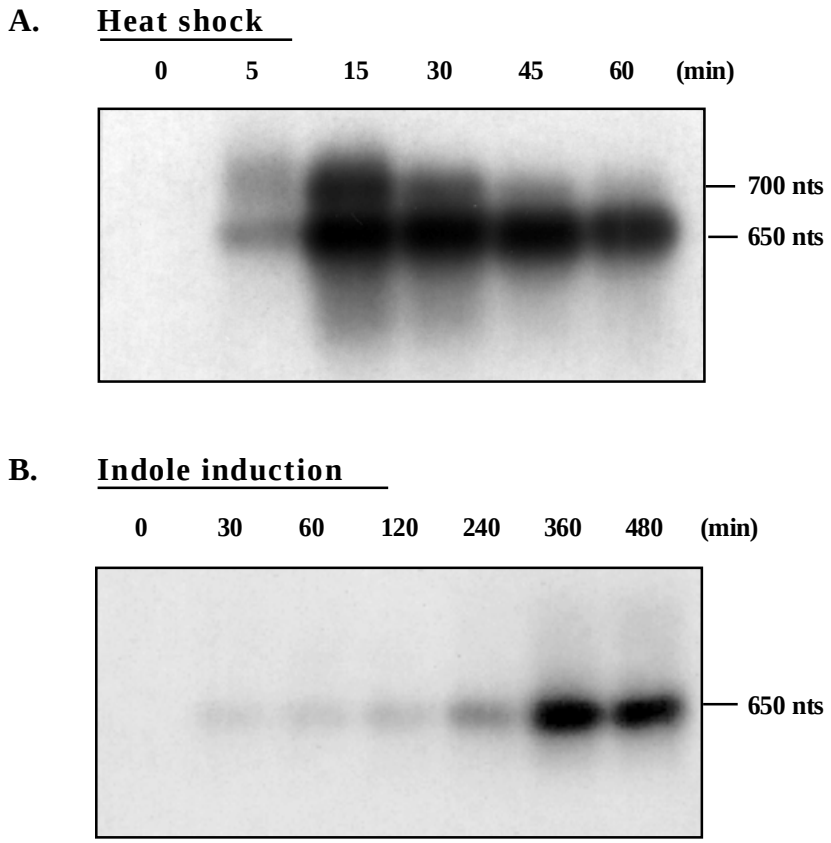


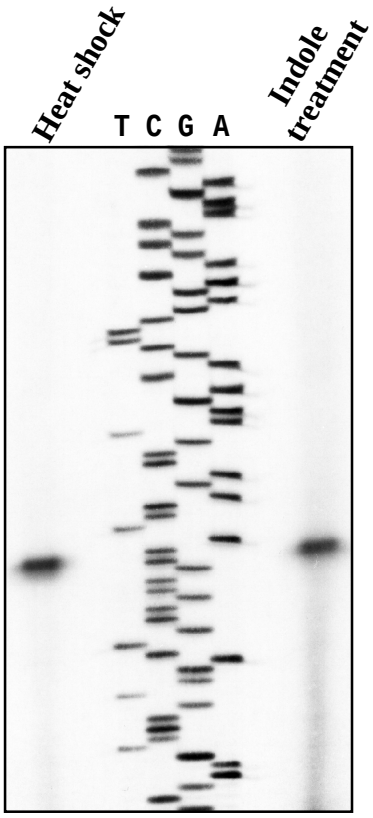
Fig. 2.7. Analysis of *hspA* transcription under stress conditions. A. Total RNA was isolated from unstressed DW4/3-1 cells and from DW4/3-1 cells 5 to 60 min after temperature shift from 28°C to 38°C. Northern hybridisation was performed using the *hspA* gene as a probe as in 2.1.1. B. Total RNA was isolated from unstressed DW4/3-1 cells and from DW4/3-1 cells 30 to 480 min after addition of indole to a final concentration of 0.5 mM. Northern hybridisation was carried out using the same probe as in A.

2.2.2. Determination of the transcription initiation site of *hspA*

The transcriptional initiation site of *hspA* during heat shock and indole treatment was determined by primer extension. Total RNA isolated from DW4/3-1 cells 15 minutes after temperature up-shift or 8 hours after addition of indole was used as template. ^{32}P-labelled oligo nucleotide R1 that is complementary to the first 20 bp of the *hspA* coding region (Fig. 2.6) was used as the reverse primer. The primer extension experiment revealed an identical transcription initiation site to be used under both stress conditions. It is located 50 bp

upstream of the translation start site of *hspA* (Fig. 2.8). No further transcription initiation site could be found even after careful reading of the autoradiography.

A.



B.

```
                -35
GAGGCCATGCCGTCTTGCGCTGTCTCGAAGCTGT
    -10         +1
CTTACGGTCTGGATGGCGGCGGCAGTCCACGGGA
                                +51
CTTCGCCGCGATACGAGAGGAGGAACCGTCATG
                  RBS           hspA
```

Fig. 2.8. Determination of the transcriptional initiation site of *hspA*. A. Primer extension analysis was performed with total RNA isolated from DW4/3-1 cells 15 minutes after the beginning of heat shock or 8 hours after addition of indole. The primer R1 is indicated in Fig. 2.6. B. The upstream sequence of *hspA*. Numbering of bases is relative to the transcriptional start site of *hspA*. Also the bp -35 and bp -10 sites are shown. RBS indicates the putative ribosome binding site.

2.3. Delimitation of the *hspA* promoter region

2.3.1. *attP-attB* mediated ectopic recombination in *S. aurantiaca*

For the determination of the minimal upstream region of *hspA* that is sufficient for the maximum expression of *hspA*, it is necessary to fuse the 5'-flanking regions of *hspA* with a reporter gene. After transferring such constructs into *S. aurantiaca* cells, the expression of the reporter gene under the control of *hspA* promoter can be measured. In bacterial genetic research, such transcriptional fusion constructs are usually carried by a vector plasmid. However, this strategy is not suitable for *S. aurantiaca*, since so far no plasmid is available that replicates in myxobacteria.

It has been shown for *M. xanthus* that an attachment site (*attP*) from myxophage Mx8 can efficiently integrate into the attachment site (*attB*) of the bacterial genome *via* a site specific recombination (Orndorff *et al.*, 1983; Tojo *et al.*, 1996). Therefore, it would be an alternative

method to introduce foreign DNA into *S. aurantiaca* cells if the *attP* site of Mx8 can also integrate into the genome of this bacterium efficiently.

Plasmid #9 (kindly provided by B. Maxl, ZMBH, Germany) was employed. It carries a 5.5 kbp *Xho*I-*Sac*I fragment from plasmid pLJS49 that contains the *attP* site and *intP* gene of Mx8 (Fig. 2.9) in the *Xho*I/*Sac*I sites of the vector pBluescript SK- (Stratagene). This fragment has been proven to integrate into the genome of *M. xanthus* efficiently (Li and Shimkets, 1988).

2.3.1.1. Construction of the indicator gene plasmid pSH8

A 3.3 kbp *Eco*RI-*Hin*dIII fragment harbouring the *rtrpA-lacZ* gene from plasmid mini Tn5 lacZ1 (de Lorenzo *et al.*, 1990) was cloned into the vector pBC SK+ (Stratagene). This reporter gene is composed of the whole *lacZ* gene and ca. 150 bp of the *trpA* gene fused to the 5'-end of *lacZ.* The *trpA* fragment has three stop codons that block translation in the three reading frames and thus completely blocks a translational fusion.

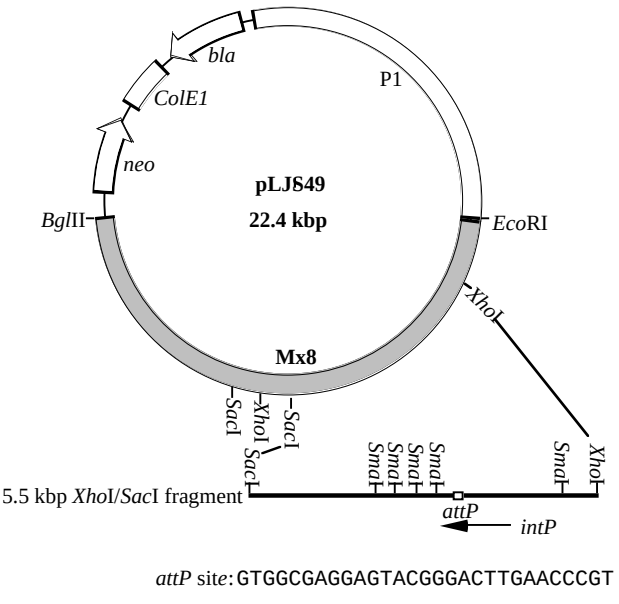


Fig. 2.9. The map of pLJS49. *ColE1*, the replication origin from *E. coli* plasmid ColE1. P1, the necessary region for P1 phage transduction. *bla*, the ampicillin resistance gene. *neo*, the kanamycin resistance gene. Mx8, the 10.5 kbp *Eco*RI-*Bgl*II fragment from Mx8 phage. The 5.5 kbp *Xho*I-*Sac*I fragment is shown below the plasmid, in which the location of *attP* site (open bar) and the orientation of the *intP* gene (arrow) are indicated. The sequence of *attP* is shown below the plasmid (reference from (Tojo *et al.*, 1996) and personal communication with P. Youderian, University of Idaho, USA).

A 1.6 kbp *Hin*dIII-*Sal*I fragment harbouring the kanamycin resistance gene (*neo*) of Tn5 from pUC4-KIXX (Pharmacia) was then cloned downstream of *rtrpA-lacZ* to generate pSH2.

A *Bam*HI-*Sma*I insert of pSH2, containing the *rtrpA-lacZ* reporter gene and the *neo* gene of Tn5, was subcloned into the *Bam*HI/*Eco*RV sites of the modified vector pSUP102, in

which the 1918 bp *Bsa*BI-*Hin*dIII fragment was replaced by a multiple cloning sites: *Bgl*I, *Eag*I, *Not*I, *Spe*I, *Kpn*I, *Sal*I, *Mlu*I, *Bam*HI, and *Hin*dIII to generate pSH3.

The multiple cloning site (*Kpn*I, *Eco*RI, *Bam*HI, *Hin*dIII, *Xba*I, *and XhoI*) was cloned into the *Kpn*I/*Xho*I sites of plasmid #9 to generate plasmid #9-MCS. The *Bam*HI-*Xba*I insert of pSH3 containing *rtrpA-lacZ* reporter gene and Tn5 *neo* gene cassette was subcloned into the *Bam*HI/*Xba*I sites of #9-MCS resulting in pSH8 (Fig. 2.10).

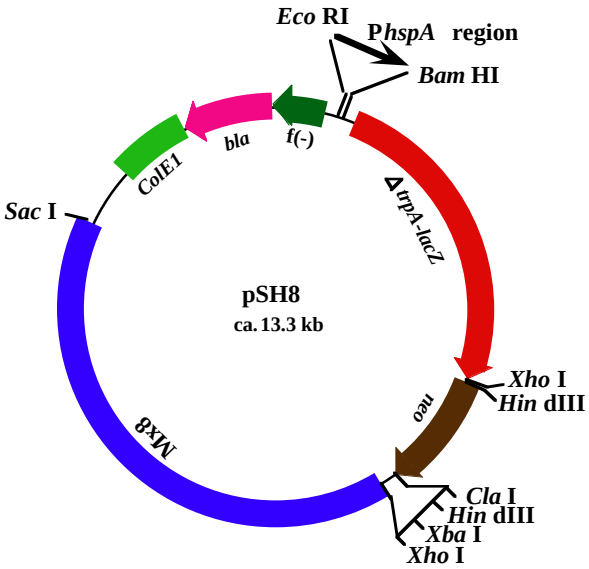


Fig. 2.10. The map of pSH8. *ColE1*, the replication origin from *E. coli* plasmid ColE1. f1 (-), the replication origin from f1 phage. *bla*, the ampicillin resistance gene. *neo*, the kanamycin resistance gene. The arrow indicates the insertion direction of the *hspA* promoter region in the following work. Other details see text.

2.3.1.2. The efficiency and specificity of *attP-attB* mediated integration into the *S. aurantiaca* chromosome

pSH8 was transferred into DW4/3-1 cells by electroporation. The transformants were selected on Tryptone agar plates containing 50 μg/ml kanamycin sulphate. The transformation efficiency was in the range of 10^5-10^6/μg supercoiled pSH8 DNA. Southern hybridisation analysis was performed with *Bam*HI/*Hin*dIII restricted genomic DNA of several positive clones selected randomly using the ^{32}P-labelled 5.5 kbp insert of plasmid #9 as a probe.

Fig. 2.11 shows that all strains have the same hybridisation pattern, suggesting the integration of the *att*P site into the genome of *S. aurantiaca* to be site-specific.

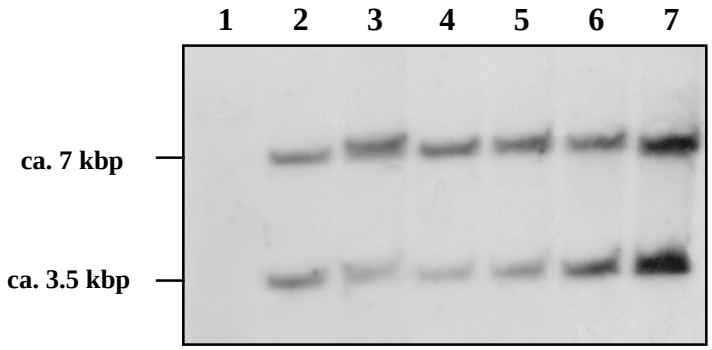


Fig. 2.11. Southern analysis of *Bam*HI/*Hin*dIII restricted genomic DNA of *S. aurantiaca* wild-type strain and 6 independent recombinant strains, in which plasmid pSH8 was inserted into the chromosome. Lane 1, 10 µg DNA isolated from DW4/3-1; lanes 2-7, 10 µg DNA isolated from 6 kanamycin resistant strains, respectively. Total DNA was digested with *Bam*HI and *Hin*dIII. Southern hybridisation was performed using the ^{32}P-labelled 5.5 kbp insert of plasmid #9.

2.3.2. The promoter activity of *hspA* at its original site and at the *attB* site

The transcriptional activity of the *hspA* promoter (P*hspA*) when fused to the *lacZ* reporter gene is reflected by the ß-galactosidase activity. To investigate whether the promoter activity of *hspA* at the *attB* site represents its activity at its original locus, strain SH2302 and SH2802 were constructed, in which the *lacZ* expression is under the control of P*hspA* at the two loci, respectively. The promoter activity of *hspA* was measured from the two strains.

2.3.2.1. Construction of plasmids pSH15, pSH33, and pSH16

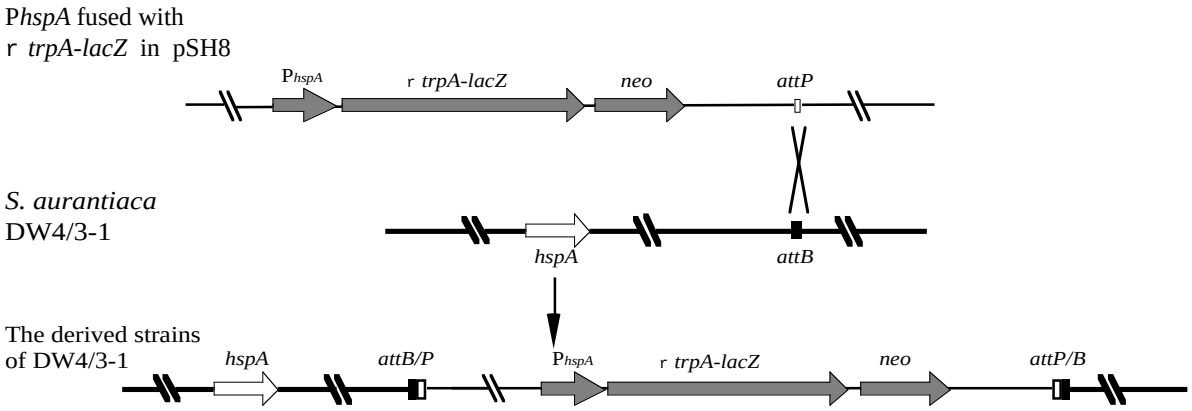
A *Cla*I-*Bam*HI fragment carrying 1252 bp of the upstream region of *hspA* and the first 65 bp of *hspA* coding region was cloned into pBC SK+, to generate pSH15. This fragment was filled-in at the *Cla*I restricted-end and cloned into the *Bam*HI and the in-filled *Eco*RI sites of pSH3 generating pSH33.

The *Cla*I-*Bam*HI insert of pSH15 was filled-in at the *Cla*I restricted-end and cloned into the *Bam*HI and the in-filled *Eco*RI sites of pSH8 to generate pSH16.

2.3.2.2. Construction of strains SH2302 and SH2802

After transferred into DW4/3-1 by electroporation, plasmid pSH16 integrated into the *attB* site resulting in strain SH2802 (Fig. 2.12A). pSH33 integrated into the upstream region of *hspA via* homologous recombination to generate SH2302 (Fig. 2.12B.).

A.



B.

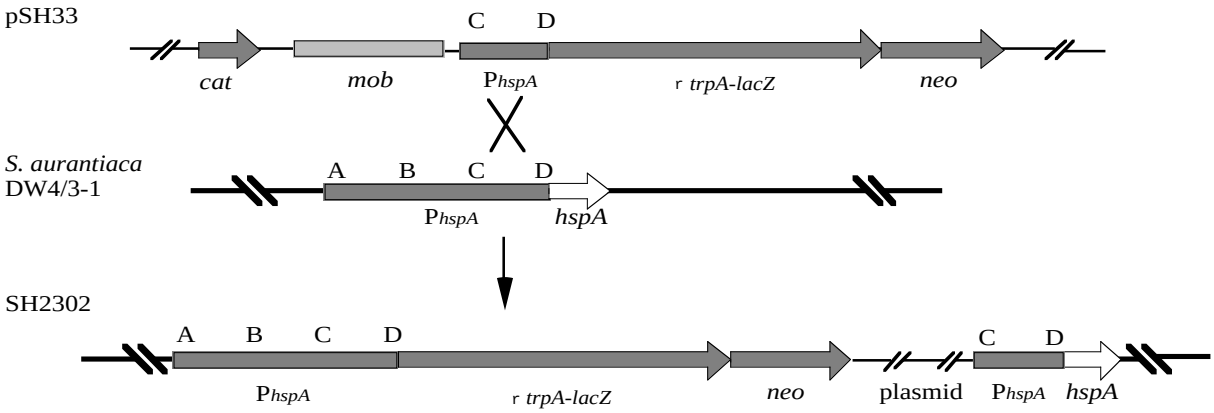


Fig. 2.12. A. Scheme of the construct of DW4/3-1 derived strains, including SH2802, in which the upstream region of *hspA* is fused to the *rtrpA-lacZ* reporter gene at the *attB* site of the genome of DW4/3-1. B. Construction of SH2302 in which pSH33 was inserted upstream of the *hspA* locus.

2.3.2.3. The promoter activity of *hspA* at the *attB* site and at its original locus

The expression of *lacZ* was under the control of the full length upstream region of *hspA* at its original locus in strain SH2302. Under heat shock conditions, expression of *lacZ* was induced rapidly in SH2302 (Fig.2.13A). ß-galactosidase activity reached a plateau 60 to 80 minutes after temperature up-shift. It was 8 to 10 folds higher than the basal level.

As expected from the Northern analysis of *hspA* transcription, ß-gaclactosidase activity increased much more slowly after the addition of indole in SH2302 (Fig. 2.13B). The maximum ß-galactosidase activity that was only half of that obtained by heat induction was obtained about 10 hours after addition of indole.

As shown in Fig. 2.13, the stressor induced synthesis of ß-galactosidase in strain SH2802 was similar in regard to kinetics and extent as in strain SH2302. This result indicates that a translocation of P*hspA* from its original position to the putative *attB* site does not affect its function.

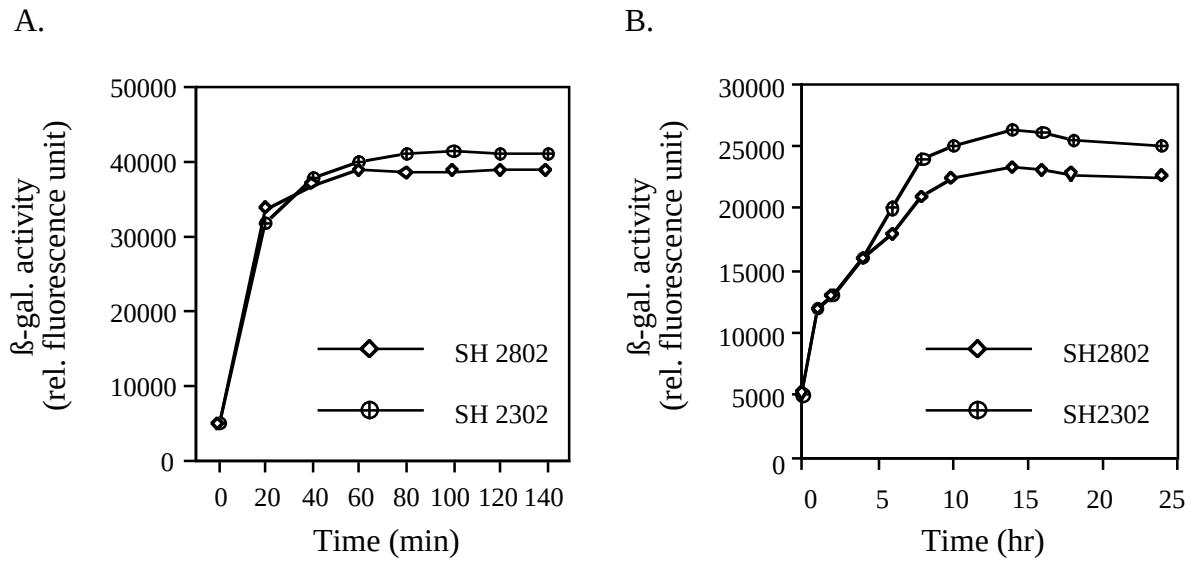


Fig. 2.13. Expression of *lacZ* in strains SH2302 and SH2802. A. Soluble protein was isolated from SH2302 and SH2802 cells before and after different time periods of heat shock. ß-galactosidase (ß-gal.) activity (presented as relative (rel.) fluorescence unit) was measured with 10 µg of soluble protein using 4-MUG as a substrate by fluorescence spectrophotometry at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. B. Soluble protein was isolated from SH2302 and SH2802 cells before and after addition of 0.5 mM indole at different time periods. ß-galactosidase activity was measured under the same condition as in A

2.3.3. Mapping of the *hspA* promoter region

To determine the promoter region of *hspA*, a subset of plasmids was constructed in which the fragments with different sizes of the upstream region of *hspA* were fused to *rtrpA-lacZ* reporter gene. In rest part of this work, the position of certain fragments or certain sites was relative to the translation start site of *hspA* assigned +1, unless otherwise indicated.

2.3.3.1. Construction of plasmids pSH28, pSH22, pSH23, pSH36, and pSH41

To generate pSH28, a *Nco*I-*Bam*HI fragment (filled-in at the *Nco*I site) ranging from bp –587 to bp +65 from the insert of pSH15 was cloned into the *Bam*HI and the in-filled *Eco*RI sites of pSH8. To generate pSH22, an *Eco*RI/*Bam*HI restricted PCR product ranging from bp -225 to bp +65 was cloned into the *Eco*RI/*Bam*HI sites of pSH8. The PCR product was obtained by amplification of pSH1 using the primer pair S4E and B1. pSH23 was constructed similarly as pSH22, as well as pSH36 and pSH41. In pSH23, the PCR product ranging from bp -379 to bp +65 was obtained by amplification of pSH1 using the primer pair E3 and B1. In pSH36, the PCR product ranging from bp -192 to bp +65 was obtained by the amplification of pSH1 using the primer pair E5 and B1. In pSH41, the PCR product ranging from bp -97 to bp +65 was obtained by the amplification of pSH1 using the primer pair S5E and B1.

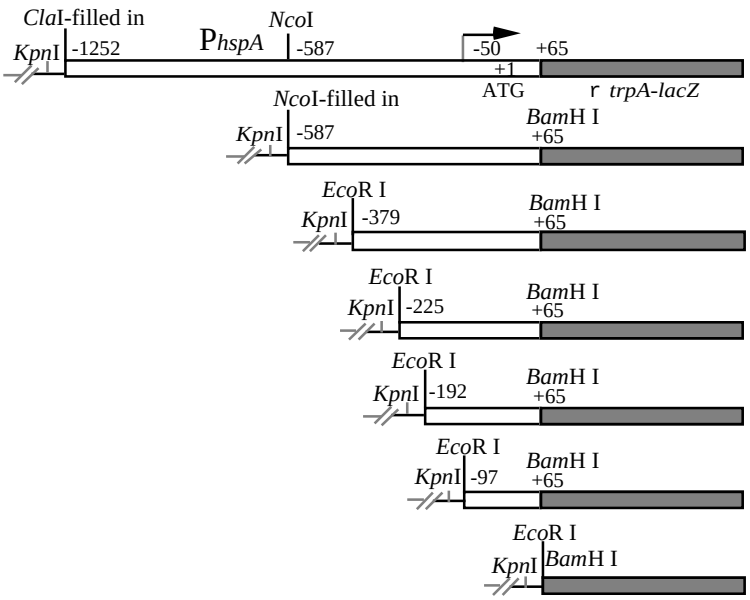
2.3.3.2. Determination of the minimal upstream region of *hspA* required for the maximum expression of *hspA* during heat shock and indole treatment

The promoter region of *hspA* was delimited by promoter mapping. Plasmids pSH8, pSH28, pSH23, pSH22, pSH36, and pSH41 were transferred into *S. aurantiaca* DW4/3-1 by electroporation. They integrated ectopically into the *attB* site, not into the upstream region of *hspA*, due to the efficient recombination between the *attP* site of these plasmids and the *attB* site of the DW4/3-1 genome (Fig. 2.12A.). Strains SH2801, SH2803, SH2804, SH2805, SH2819, and SH2821 were generated in this way (Fig. 2.14). The structure of recombinant strains was confirmed by Southern hybridisation analysis (data not shown).

Fig. 2.14 shows that the *lacZ* expression in strains SH2802, SH2803, SH2804, and SH2805 did not show significant difference upon heat stimulation. This indicates that the deletion from bp -1252 to bp -225 does not impair the promoter activity of *hspA*. In strain SH2819, only 192 bp upstream region of *hspA* was fused to *lacZ* gene, which led to a reduction of the *lacZ* expression by half. In strain SH2821, only 97 bp upstream region of *hspA* was fused to *lacZ* gene. No increased ß-galactosidase synthesis was obtained during heat shock. The same result was obtained with strain SH2801. Taken together, these results indicate that the 225 bp upstream sequence of *hspA* is sufficient for the maximum expression of *hspA* in response to heat shock.

During indole treatment, the expression of *lacZ* in strain SH2802 was observed as the same as in strain SH2803. The reduction of the *hspA* upstream region in strains SH2804, SH2805, and SH2819 impaired the expression of *lacZ*. The results of these experiments

indicated that the 587 bp upstream sequence of *hspA* is required for the maximum expression of *hspA* in response to indole induction. In SH2821 as well as in SH2801, the expression of *lacZ* is extinguished, as observed under heat shock conditions.



ß-gal. activity (rel. fluorescence)

Strains	Before induction	Heat shock (80 min)	Indole induction (14 hr)
SH2802	5200	39000	23300
SH2803	4500	38500	20000
SH2804	4400	35400	10400
SH2805	4000	35800	9700
SH2819	2850	19000	6000
SH2821	2300	2400	2320
SH2801	1900	1900	2200

Fig. 2.14. The promoter mapping assays of *hspA*. Soluble protein was isolated from strains SH2802, SH2803, SH2804, SH2805, SH2819, SH2821, and SH2801 before and 80 minutes after temperature up-shift from 28°C to 38°C or 14 hours after addition of 0.5 mM indole. ß–galactosidase activity was measured as described in 2.3.2.3.

2.3.4. *hspA* transcription in a σ^B mutant strain

The housekeeping sigma factor of *S. aurantiaca* DW4/3-1 is σ^A that is expressed at any stage of the cell cycle. There are two other sigma factors known to be expressed under heat shock conditions in this bacterium: one is σ^B and the other one is an unknown sigma factor (personal communication with B. Silakowski, ZMBH, Germany). To elucidate whether *hspA* is transcribed by an RNA polymerase bearing σ^B, the transcription of *hspA* was examined in a σ^B mutant strain, BS53 (kindly provided by B. Silakowski, ZMBH, Germany), under heat shock conditions.

Northern hybridisation was performed with total RNA isolated from vegetative and heat shocked BS53 cells at different time periods after temperature up-shift using ^{32}P-labelled *hspA* PCR product as a probe (see 2.1.1). Total RNA isolated from 10 minutes heat shocked

wild–type DW4/3-1 cells was analysed as a control. Fig. 2.15 shows that the transcription of *hspA* in BS53 was the same as in DW4/3-1 wild-type strain, indicating that σ^B does not control the transcription of *hspA* under heat shock conditions.

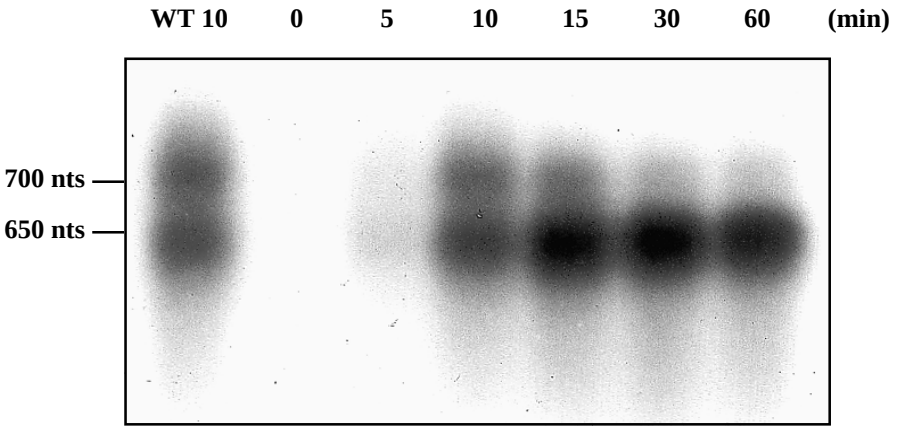


Fig. 2.15. Northern hybridisation analysis of the *hspA* expression in BS53. Total RNA was isolated from BS53 before and after heat shock at different time periods (5 to 60 minutes). Total RNA isolated from DW4/3-1 cells 10 minutes after temperature up-shift was used as a control. Northern hybridisation was performed as in 2.2.1.

2.4. Transcriptional regulation of the *hspA* expression under heat shock conditions

To understand how the expression of *hspA* is regulated at the transcription level after heat stimulation, the putative regulatory elements involved in the promoter activity of *hspA* were investigated.

2.4.1. Confirmation of the -10 and -35 regions within P*hspA*

Primer extension experiments showed that the transcription of *hspA* starts at 50 bp upstream of the *hspA* start codon. To prove that this is the real transcriptional start site of *hspA*, four plasmids were constructed in which different mutations were introduced within the deduced -10, -35 regions of P*hspA*. The mutated promoter regions were fused to the *rtrpA-lacZ* gene.

2.4.1.1. Construction of plasmids pSH31 and pSH32

Plasmid pSH31 was constructed by cloning an *Eco*RI/*Bam*HI restricted PCR fragment that carries the upstream region of *hspA* from bp -379 to bp -95 into the *Eco*RI/*Bam*HI sites of

pSH8. The PCR fragment was generated with the primer pair E3 and B2 using pSH1 as template.

Plasmid pSH32 was constructed by cloning an *Eco*RI/*Bam*HI restricted PCR fragment that carries the upstream region of *hspA* from bp -379 to bp -125, and was generated with the primer pair E3 and B3 using pSH1 as template.

2.4.1.2. Construction of plasmids pSH25 and pSH34

To construct pSH25, a PCR product carrying the upstream region of *hspA* from bp -379 to bp -86 was amplified with the primer pair E3 and C12 using pSH1 as template. The fragment was cloned into the *Eco*RI/*Cla*I sites of the vector pBR322 (New England Biolab) to generate pSH25A. Another PCR product ranging from bp -85 to bp +65 was generated with the primer pair C11 and B1, and cloned into the *Cla*I/*Bam*HI sites of pSH25A to generate pSH25B. An additional *Cla*I hexamer was thus inserted between bp -86 and bp -85 in the *Eco*RI-*Bam*HI insert of pSH25B that ranges from bp -379 to bp +65. This fragment was subcloned into pSH8 resulting in pSH25.

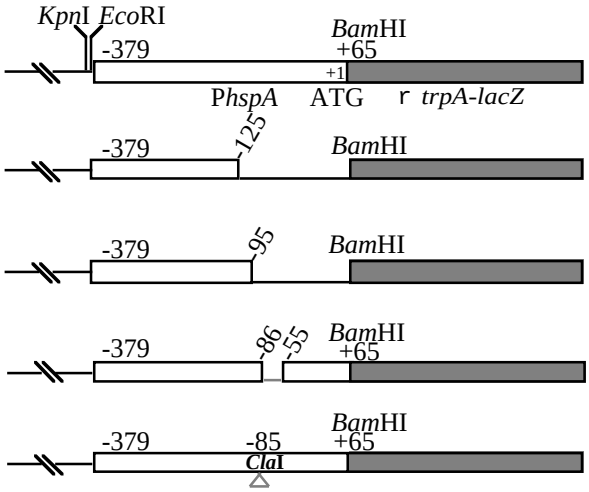
pSH34 was constructed similarly as pSH25. In pSH34A, the PCR product ranging from bp -379 to bp -86 was generated with the primer pair E3 and C12. In pSH34B, the second PCR product ranging from bp -55 bp +65 was generated with the primer pair C13 and B1. The *Eco*RI-*Bam*HI insert of pSH34B that ranges from bp -379 to bp +65 of which the sequence from bp -85 to bp -56 was deleted was subcloned into pSH8 resulting in pSH34.

2.4.1.3. Construction of strains SH2812, SH2814, SH2815, and SH2817 and the analysis of *lacZ* expression in these strains

Plasmids pSH25, pSH31, pSH32, and pSH34 were transferred into DW4/3-1 cells by electroporation. The integration of these plasmids into the *attB* site of DW4/3-1 genome generated strains SH2812, SH2814, SH2815, and SH2817 (Fig. 2.16).

Determination of ß-galactosidase activity showed that the removal of the upstream sequences of *hspA* ranging from bp -124 to bp +65 (strain SH2815), from bp -94 to bp +65 (strain SH2814) and from bp -85 to bp -56 (strain SH2817) resulted in the loss of *hspA* expression (Fig. 2.16). According to the result of primer extension (2.2.2), these deleted regions contain the putative -35, -10 regions of P*hspA*. In strain SH2817, only bp -35 to bp -6 (relative to the putative transcription start site of *hspA*) upstream sequence of *hspA* was deleted. The expression of *lacZ* was reduced to the basal level. These results, in accordance with the primer extension results indicate that the bp -85 to bp -56 upstream region of *hspA* carries the holoenzyme RNA polymerase binding site. Insertion of 6 bp between bp -86 and

bp -85 (strain SH2812) did not affect the heat dependent expression of *hspA*. This suggests that the -35 region of P*hspA* is not in the centre of bp -35.



ß-gal. activity (rel. fluorescence unit)

Strains	Before induction	Heat shock (80 min)
SH2804	4400	35400
SH2815	2500	2600
SH2814	2500	2550
SH2817	2600	2650
SH2812	5000	33200

Fig. 2.16. Mutational analysis of the deduced -10 and -35 regions of P*hspA*. The mutated regions are shown in the figure. Soluble protein was isolated from strains SH2804, SH2815, SH2814, SH2817, and SH2812 before and 80 minutes after the temperature up–shift from 28°C to 38°C. ß-galactosidase activity was measured as in 2.3.2.3.

2.4.2. Screening of putative regulatory elements within P*hspA*

To find out whether transcription regulator(s) are involved in the transcriptional regulation of *hspA* under heat shock conditions, gel mobility shift assays were performed. DNA fragments within the P*hspA* were incubated with the cell extracts from unstressed and heat shocked DW4/3-1 cells and analysed by native polyacrylamide gel electrophoresis.

2.4.2.1. A putative regulatory region within P*hspA*

Two large DNA fragments were chosen to perform the gel shift assay. They range from bp -380 to bp -228 (G1) and from bp -240 to bp -71 (G2), respectively (see Fig. 2.6). The gel retardation experiment showed that G2 but not G1 was retarded after incubation with cell extracts from both unstressed and heat shocked cells of DW4/3-1 (Fig. 2.17A, G1 not shown). Moreover, the signal of retarded G2 is weak after incubation with cell extracts from unstressed cells and it becomes much stronger after incubation with cell extracts from heat shocked cells. This indicates that G2 can bind certain protein(s) from the cell extracts, and the binding is enhanced by heat shock. Two retarded bands of G2 were observed in Fig. 2.17A,

suggesting that more than one binding site exist in this region. They might interact with one or more proteins. The size of G2 was reduced by deletion from both 5'- and 3'-ends (data not shown) resulting in G3, which ranges from bp -223 to bp -141. G3 has the same capability to bind to the heat shock dependent protein(s) as G2 (Fig. 2.17B). According to the result of promoter mapping of *hspA* (2.3.3), since the region from bp -223 to bp -141 is required for the maximum activity of P*hspA* under heat shock conditions, it is reasonable to speculate that the G3 region carries a regulatory element involved in the P*hspA* activity.

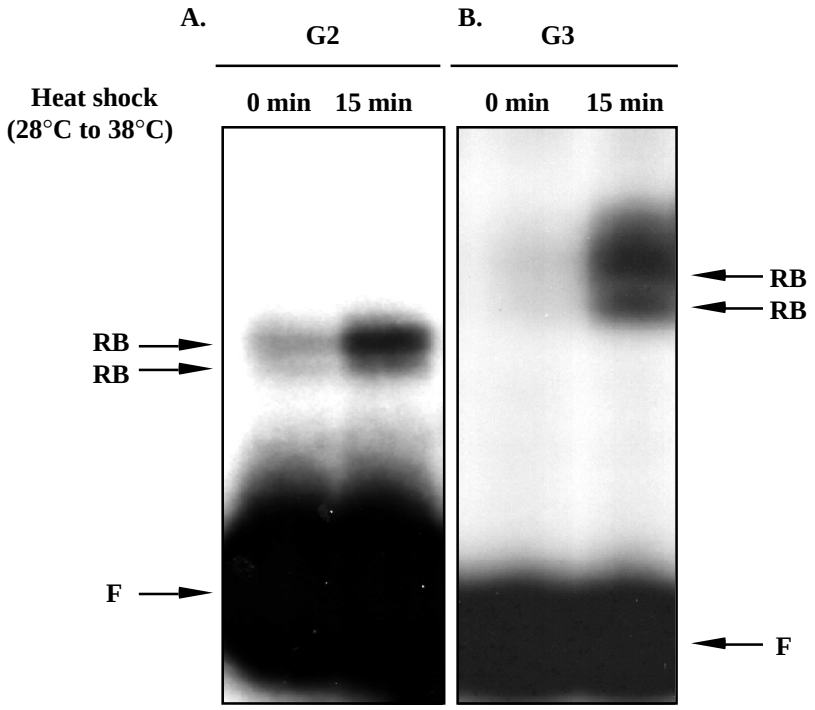


Fig. 2.17. Investigation of a putative regulatory region within P*hspA* by gel shift assays. Cell extracts were prepared from DW4/3-1 cells before and 15 minutes after the beginning of heat shock as indicated in the figure. Poly (dI-dC) was used as a general DNA competitor. A. The gel shift experiment of ^{32}P–labelled G2. B. The gel shift experiment of ^{32}P-labelled G3. Gel retardation was performed on a 4.2% native polyacrylamide gel. "RB" refers to the retarded bands, while "F" corresponds to free DNA.

To confirm that G2 and G3 carry the same binding site(s) and thus bind the same protein(s), competition gel shift assays were performed with G2 and G3. The result (Fig. 2.18) showed that when either unlabelled G2 or G3 was added in 200 fold molar excess to

^{32}P–labelled G2 (lanes 2 and 3, respectively) or G3 (lanes 5 and 6, respectively) in the binding reaction, the signal of retarded ^{32}P-labelled G2 or G3 was significantly reduced. G2 and G3 behaved as competitive DNA for each other, confirming that G2 and G3 carry the same binding site(s). The binding of the putative regulator(s) to G2 and G3 is sequence specific. Moreover, as a control, G1 could not affect the retardation of ^{32}P-labelled G2 or G3 (lanes 1 and 4).

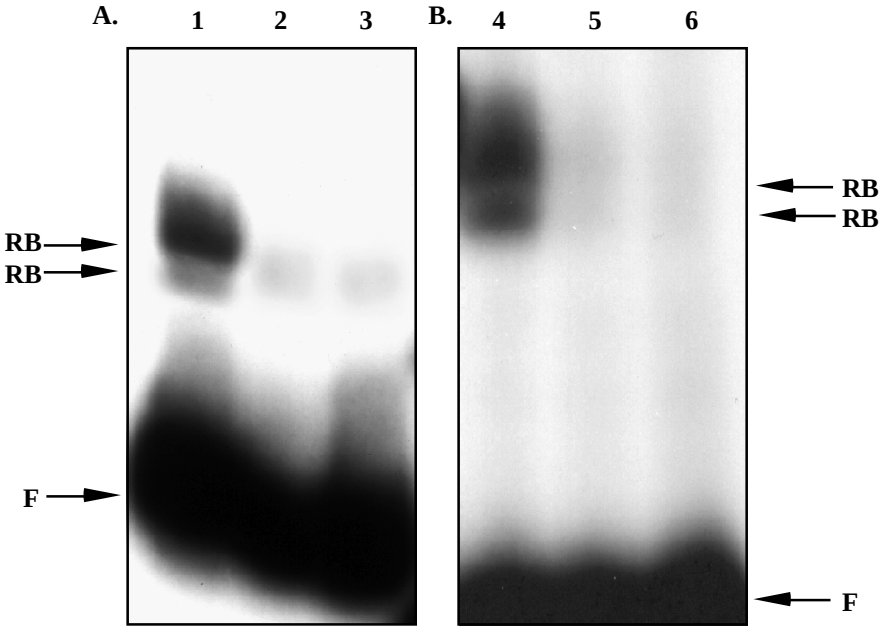


Fig. 2.18. Competition among G1, G2, and G3 analysed by gel shift assay. Cell extracts were prepared from DW4/3-1 cells 15 minutes after heat shock. A. ^{32}P-labelled G2 competed with 200 fold molar excess unlabelled G1 (lane 1), G2 (lane 2), and G3 (lane 3). B. ^{32}P-labelled G3 competed with 200 fold molar excess unlabelled G1 (lane 4), G2 (lane 5) and G3 (lane 6). "RB" and "F" have the same meaning as in Fig. 2.17.

2.4.2.2. The influence of heat shock and phosphorylation on binding of the putative regulator(s) to P*hspA*

Fig. 2.17 unequivocally proves that the intensity of the shifted bands of G2 or G3 increased if the labelled fragment was incubated with the cell free extracts of heat shocked cells. The activity of many transcription activators is achieved by phosphorylation. To investigate whether this is true in the case of *hspA* transcription, phosphorylation experiments were performed.

Cell extracts from unstressed DW4/3-1 cells or the DW4/3-1 cells that were subjected to heat shock for 5 or 15 minutes were incubated with 50 mM acetyl phosphate at 37°C for one hour before addition of ^{32}P-labelled DNA fragment G3. The gel retardation assay was

performed thereafter. As shown in Fig. 2.19, the signal of retarded G3 was increased significantly after incubation with acetyl phosphate. This suggests that phosphorylation increases the binding affinity of the putative regulator(s) to G3. As a control, when cell extracts from unstressed DW4/3-1 cells were incubated at 37°C for one hour in the absence of acetyl phosphate and then incubated with ^{32}P-labelled G3 in gel shift assay, no increase in signal intensity was observed (Fig. 2.19, lane 2). This indicates that the increase of the binding affinity of the putative regulator(s) to G3 is mainly due to the phosphorylation but not due to the 37°C incubation of the cell extracts.

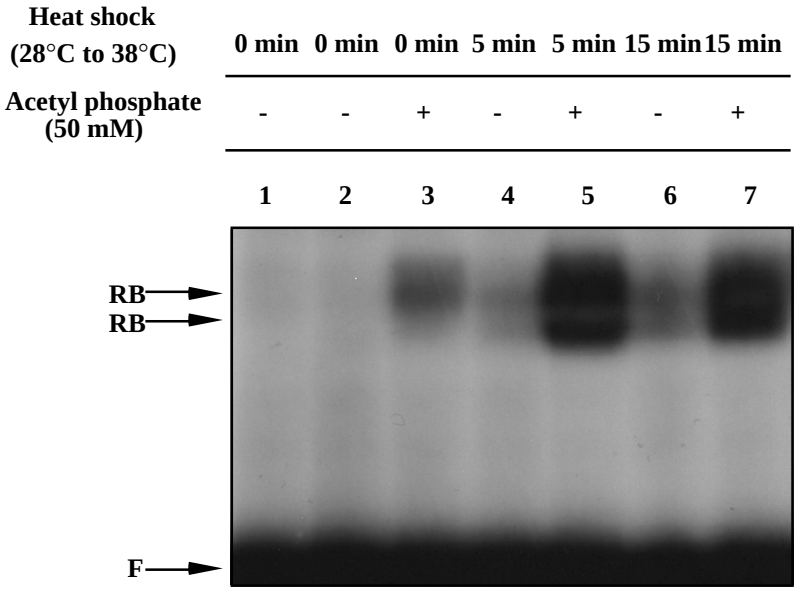


Fig. 2.19. The influence of phosphorylation on the binding affinity of the putative regulator(s) to G3. The gel shift assay was performed with ^{32}P-labelled G3. Cell extracts were isolated from unstressed or heat shocked DW4/3-1 cells as indicated in the figure. Prior to addition of ^{32}P-labelled G3, cell extracts were incubated at room temperature for 10 minutes (lanes 1, 4, 6) without acetyl phosphate, or at 37°C for one hour without acetyl phosphate (lane 2), or at 37°C for one hour with 50 mM acetyl phosphate (lanes 3, 5, 7). “RB” and “F” have the same meaning as in Fig. 2.17.

2.4.3. Structural and functional analysis of P*hspA* by mutagenesis

To find out how the putative regulatory element (G3) is involved in the transcriptional regulation of *hspA* and whether there are other regulatory elements involved in the transcriptional regulation of *hspA* under heat shock conditions, P*hspA* has been further studied

by deletion analyses. A series of plasmids were constructed in which different deletions were introduced in the upstream sequence of *hspA*. The modified upstream regions of *hspA* were subsequently fused to the *rtrpA-lacZ* reporter gene. After integration of these plasmids into the *attB* site of DW4/3-1, the expression of *lacZ* under the control of the mutated P*hspA* was analysed.

2.4.3.1. Influence of the first 65 bp of *hspA* coding region on *hspA* expression

To elucidate whether the first 65 bp coding sequences of *hspA* are involved in the expression of *hspA*, plasmid pSH42 was constructed by cloning an *Eco*RI/*Bam*HI restricted PCR fragment into the *Eco*RI/*Bam*HI sites of pSH8. This PCR fragment carries the 225 bp upstream region of *hspA*, which was generated with the primer pair S4E and B4 using pSH1 as template.

Integration of pSH42 into the *attB* site of DW4/3-1 resulted in strain SH2822. Strains SH2805 (Fig. 2.14) and SH2822 (Fig. 2.20) showed the same ß-galactosidase activity after heat shock. This indicates that the first 65 bp coding sequence of *hspA* has no influence on *hspA* transcription. But this does not exclude this region to influence translation in appropriate strains.

2.4.3.2. The function of the G3 region

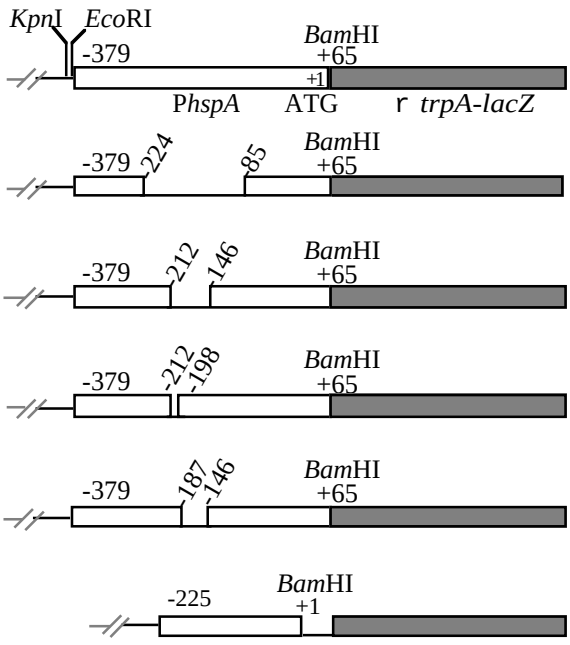
To analyse the contribution of G3 to *hspA* expression, mutants SH2816 and SH2820 that harbour deletions in the ectopically integrated *hspA* promoter region were constructed (Fig. 2.20). They were obtained by integration of the plasmids pSH29 and pSH30 into the *attB* site of the wild-type DW4/3-1 strain.

To construct pSH29, the upstream region of *hspA* from bp -379 to bp -212 was amplified with the primer pair E3 and C2 using pSH1 as template. The PCR product was cloned into the *Eco*RI/*Cla*I sites of pBR322 to generate pSH29A. Another DNA fragment ranging from bp –146 to bp +65 was generated with the primer pair C9 and B1 using pSH1 as template. The resulting fragment was cloned into the *Cla*I/*Bam*HI sites of pSH29A to generate pSH29B. The *Eco*RI-*Bam*HI insert of pSH29B that ranges from bp -379 to bp +65 harbouring a deletion from bp -211 to bp -147 was subcloned into pSH8 to generate pSH29.

Plasmid pSH30 was constructed similarly. Using the primer pair E3 and C14, the upstream region of *hspA* from bp -379 to bp -224 was amplified and cloned into the *Eco*RI/*Cla*I sites of pBR322 to generate pSH30A. The sequence from bp -85 bp +65 was amplified using the primer pair C11 and B1 and cloned into the *Cla*I/*Bam*HI sites of pSH30A resulting in pSH30B. The *Eco*RI/*Bam*HI insert of pSH30B was subcloned into pSH8 to

generate pSH30, which contains a fragment ranging from bp -379 to bp +65 with a deletion that ranges from bp -223 to bp -86.

Fig. 2.20 shows that the maximum ß-galactosidase activity induced by heat shock in strain SH2816 is only 38% of that obtained in strain SH2804. In strain SH2816, most of the putative regulator binding region, G3, is deleted. This suggests the putative regulator(s) that binds to G3 to be transcription activator(s).



ß-gal. activity (rel. fluorescence unit)

Strains	Before induction	Heat shock (80 min)
SH2804	4400	35400
SH2820	3400	5200
SH2816	2800	14500
SH2813	4500	21500
SH2811	4800	24500
SH2822	3700	33800

Fig. 2.20. Transcriptional activity analysis of mutated P*hspA*. The deletion region of each construct is indicated in the figure. Soluble protein was isolated from strains SH2820, SH2816, SH2813, SH2811, and SH2822 before and 80 minutes after temperature up-shift from 28°C to 38°C. ß-galactosidase activity was determined as in 2.3.2.3. Strain SH2804 was used as a control.

Surprisingly, the heat shock induced maximum ß-galactosidase activity of strain SH2820 was reduced to only about 6% of that determined in strain SH2804 (Fig. 2.20). The different deletion effect between SH2820 and SH2816 suggests that a *cis*-acting element should exist just upstream of the -35 region in P*hspA* .

2.4.3.3. The putative regulator binding site(s) on G3

The DNA fragment G3 contains two inverted repeats (Fig. 2.6). One of them is located from bp -214 to bp -196 with the sequence: 5'-CCGCCTGN$_5$CAGGCGG-3' or alternatively from bp -213 to bp -185 with the sequence: 5'-CGCCTGN$_{17}$CAGGCG-3'. The second one is located between bp -165 and bp -144 with the sequence: 5'-CCCTTN$_{12}$AAGGG-3'. One might speculate that these inverted repeats are the binding sites for the transcription activator(s).

To analyse the influence of the inverted repeats of G3 on the transcriptional activation of P*hspA*, plasmids pSH24 and pSH26 were constructed in which the inverted repeat regions were deleted.

The upstream region of *hspA* from bp -379 to bp -187 was amplified using the primer pair E3 and C6 and cloned into the *Eco*RI/*Cla*I sites of pBR322 resulting in pSH24A. The region from bp -146 to bp +65 was amplified using the primer pair C9 and B1 and cloned into the *Cla*I/*Bam*HI sites of pSH24A to generate pSH24B. The *Eco*RI-*Bam*HI insert of pSH24B that renges from bp -379 to bp +65 containing a deletion ranging from bp -186 to bp -147 was subcloned into pSH8 resulting in pSH24.

In the same way, pSH26 was constructed that contains the region from bp -379 to bp +65 harbouring a deletion from bp -211 to bp -199. The primer pairs used for generating this fragment were E3 and C2 or C3 and B1, respectively.

Integration of plasmids pSH24 and pSH26 into the *attB* site of *S. aurantiaca* DW4/3-1 resulted in strains SH2811 and SH2813 (Fig. 2.20).

In strain SH2813, a part of the first inverted repeat was deleted. This led to about 55% reduction of the heat induced maximum ß-galactosidase activity. In promoter mapping assay (2.3.3), deletion of the upstream sequence of *hspA* to bp -192 (SH2819) resulted in the same reduction (about 50%, Fig. 2.14).

Deletion of the second inverted repeat (SH2811) reduced the heat induced maximum ß–galactosidase activity to about 65%.

Neither of the two deletions (SH2811 and SH2813) decreased the maximum ß–galactosidase activity to the same extent as the deletion covering both inverted repeats (SH2816). If one assumes that there is no synergistic interaction between both inverted repeats, the maximum ß-galactosidase activity of strain SH2816 should be reduced to about 36% (product of the reduction in strains SH2811 and SH2813). This agrees quite well with the maximum ß-galactosidase activity measured in strain SH2816 (38%).

2.5. Summary of the transcriptional regulation of *hspA* gene

The *hspA* gene has a monocistronic structure. Transcription of *hspA* is rapidly induced by heat shock. A maximum transcription level of *hspA* is reached during the first 15 minutes after temperature up-shift. Two transcripts of *hspA* were detected in response to heat stimulation, one is 650 nts, and the other one is 700 nts in size. Transcription of *hspA* is induced much more slowly after indole treatment. Only the 650 nts transcript of *hspA* was detected. The maximum amount of the gene product as measured by ß-galactosidase activity reached about 10 hours after addition of indole.

The transcription of *hspA* starts at the same site after heat shock and indole induction. Promoter mapping experiments suggest that the *hspA* promoter regions required under both conditions were not identical. The promoter region needed for heat shock dependent maximum expression of *hspA* extends to bp -225. Interestingly, the 587 bp upstream sequence of *hspA* is required for maximum expression of *hspA* after indole treatment.

At least three regulatory regions are involved in the activation of P*hspA* after temperature up-shift. The first region identified spans from bp -56 to bp -85 that contains the RNA polymerase binding site. Deletion of this region completely inactivates P*hspA*. The second region ranges from bp -141 to bp -223. It carries more than one putative transcription activator binding sites. Binding of the putative transcription activator(s) depends on heat shock and phosphorylation. Deletion of the second region reduces the P*hspA* activity more than 50%. Deletion of a third region that ranges from bp -86 to bp -141 together with the second region reduced the P*hspA* activity to about 6%. This suggests that a *cis*-acting element exists just upstream of the -35 region of P*hspA*. Taken together, the transcription of *hspA* seems to be mainly positively regulated under heat shock conditions.

Part II. The function of the HspA protein

2.6. Investigation of the physiological function of HspA *in vivo*

To understand the physiological function of HspA, the chromosomal *hspA* gene of *S. aurantiaca* was deleted. The phenotype of the *hspA* null mutant was analysed and compared with that of the wild-type strain.

2.6.1. Construction of a *hspA* null mutant strain

In *S. aurantiaca*, the *hspA* gene was replaced by the *neo* gene from Tn5. Both flanking regions of *hspA* were first cloned to the 3'- and 5'-ends of the *neo* gene, respectively. The plasmid harbouring this construct was amplified in *E. coli*. Subsequently, the construct was transferred into DW4/3-1 cells. The *neo* gene was integrated into the genome of DW4/3-1 *via* double homologous recombination between the 5'- and 3'-flanking regions of *hspA*. Thus, *hspA* was eliminated from the genome.

2.6.1.1. Construction of pSH6

The 1125 bp 5'-flanking region of *hspA* was amplified by PCR using the primer pair KO-*Eco*RI and KO-*Cla*I (Fig. 2.6). After *Eco*RI and *Cla*I digestion, the PCR product was cloned into the *Eco*RI/*Cla*I sites of pBR322 to generate pSH6A.

Similarly, the 1036 bp 3'-flanking region of *hspA* was amplified using the primer pair KO-*Bam*HI and KO-*Sal*I (Fig. 2.6). After *Bam*HI/*Sal*I restriction, the fragment was cloned into *Bam*HI/*Sal*I sites of pSH6A to generate pSH6B.

Finally, a ca. 1.2 kbp *Hin*dIII-*Sma*I fragment from pUC4-KIXX harbouring the *neo* gene of Tn5 was cloned into the *Cla*I/*Bam*HI sites of pSH6B that are located between the 5'- and 3'-flanking regions of *hspA* resulting in pSH6 (Fig. 2.21).

2.6.1.2. Construction of strain SH1

pSH6 was transferred into *S. aurantiaca* DW4/3-1 by electroporation. Selection for kanamycin resistance resulted in recombinants between the chromosomal DNA and pSH6 that were generated either by a single crossover (2 types) or by a double crossover (1 type). A single crossover generates merodiploid mutants that contain additionaly the plasmid. To construct mutants that are generated mainly by a double recombination event, the plasmid had to be linearised. Thus, pSH6 was digested by *Ase*I and *Nde*I. Two fragments were generated. The bigger one is about 6.5 kbp in length containing the flanking regions of *hspA* and the *neo*

gene; the size of the other one is 1.2 kbp harbouring a part of the pBR322 DNA. The bigger fragment of pSH6 was recovered from the agarose gel after electrophoresis.

In general, linear DNA is easily digested by enzymes after being introduced into bacterial cells. To protect the linear DNA, the 5'-ends of the bigger fragment from pSH6 was filled-in with dNTPαS (2'-deoxyribonucleoside 5'-O-(1-thiotriphosphates)) using the Klenow I fragment. These triphosphate analogues are very slowly hydrolysed by nucleases, e.g. exonuclease (Eckstein and Gish, 1989).

After transferring of the dNTPαS in-filled 6.5 kbp fragment into DW4/3-1 by electroporation, positive clones were selected on Tryptone medium containing 50 μg/ml kanamycin sulphate. Four kanamycin resistant clones were obtained. Theoretically, such clones would be *hspA* deletion mutants designated SH1 (Fig. 2.21) in which the entire coding region of *hspA* was replaced by the *neo* gene.

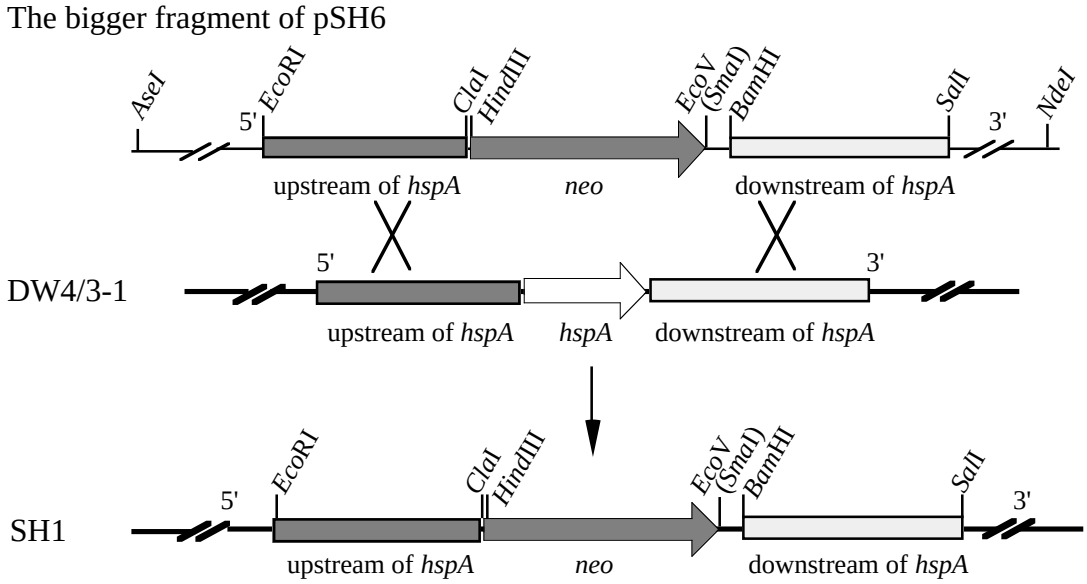


Fig. 2.21. A scheme of the construction of the *hspA* null mutant, SH1, in which *hspA* gene was replaced by the *neo* gene *via* a double crossover.

2.6.1.3. Confirmation of the *hspA* deletion in strain SH1 by Southern and Western analyses

The gene type of SH1 was examined by Southern analysis and the phenotype of SH1 was examined by Western analysis.

Southern hybridisation was performed with the *Pst*I restricted genomic DNA isolated from SH1 and DW4/3-1. A unique *Pst*I site is located in the *neo* gene. When using the ^{32}P–labelled 5.5 kbp *Pst*I fragment from pSH1 as a probe that carries the *hspA* gene and its

flanking regions, two *Pst*I restriction fragments were detected with SH1 DNA (Fig. 2.22A). This is due to the replacement of *hspA* by the *neo* gene. Only the 5.5 kbp *Pst*I fragment was detected after *Pst*I restriction of DW4/3-1 DNA. Furthermore, when using the ^{32}P-labelled 1.2 kbp *Hin*dIII/*Sma*I fragment from pUC4-KIXX that harbours the *neo* gene of Tn5 as a probe, no restriction fragment from DW4/3-1 genomic DNA was detected. As expected, two fragments were detected in *Pst*I restricted genomic DNA of SH1 (Fig. 2. 22B).

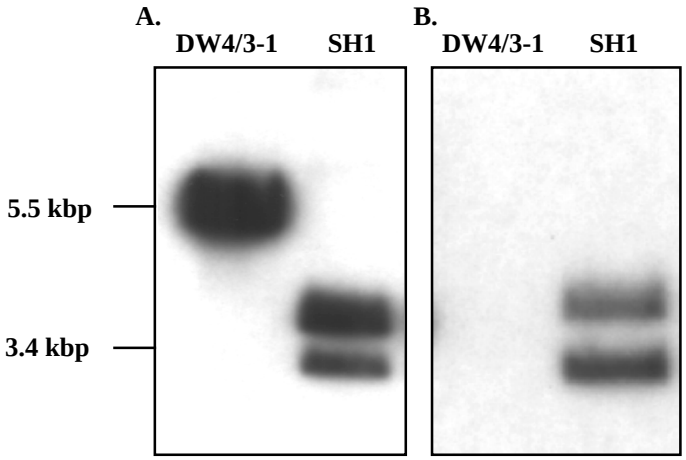


Fig. 2.22. Southern analysis of SH1 genomic DNA. A. *Pst*I restricted genomic DNA from SH1 and DW4/3-1 was hybridised with the ^{32}P–labelled 5.5 kbp *Pst*I fragment from pSH1. B. *Pst*I restricted genomic DNA from SH1 and DW4/3-1 was hybridised with the ^{32}P–labelled 1.2 kbp *Hin*dIII-*Sma*I fragment from pUC4-KIXX harbouring the *neo* gene.

Western hybridisation was performed with the cell free extracts from SH1 cells before and after temperature up-shift and with cell extracts from DW4/3-1 cells that have been subjected to 90 minute heat shock treatment. The purified recombinant $HspA_{His}$ protein (see 2.7.1) was used as a control. Fig 2.23 shows that HspA was no more synthesised in SH1 cells before and after heat shock. This proved *hspA* to be deleted in strain SH1.

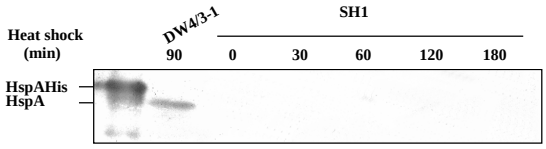


Fig. 2.23. Western analysis of HspA synthesis in DW4/3-1 and SH1. Cell free extracts from heat shocked DW4/3-1 cells, from unstressed and heat shocked SH1 cells were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel. 2 µg of pure recombinant $HspA_{His}$ protein was loaded onto the gel as a control. Anti-HspA-Sera was diluted to 1:10,000.

2.6.2. Thermotolerance of SH1

As HspA is an abundant protein in heat shocked cells and myxospores of *S. aurantiaca* DW4/3-1, it was speculated that this polypeptide plays a role in thermotolerance and cell differentiation. Therefore, both vegetative and developmental cell cycles including aggregation, stalk formation, fruiting body formation, sporulation, and germination of SH1 were examined. DW4/3-1 was examined in parallel as a control.

Cell growth of SH1 and DW4/3-1 was tested at normal and elevated growth temperatures. Cells were grown at 32°C until middle log phase was reached (1×10^8 cells/ml). Then the cells were inoculated in fresh Trypton medium containing 120 μg/ml streptomycin sulphate to a cell density of about 10^6 cells/ml. The cell culture was then divided into several portions of 50 ml and incubated at 32°C, 35°C, and 37°C, respectively. After 48 hours of growth, cell density of each portion was measured. Table 2.1 shows that the cultures of SH1 and DW4/3-1 grown at 32°C and 35 °C have similar growth rates. The cell density reached 10^8 cells/ml after 48 hours. At 37°C, the growth rate of *S. aurantiaca* cells was reduced. Cell density reached about 3×10^7cells/ml after 48 hours, and 10^8 cells/ml after 56 hours. Some myxospores were observed in these cell cultures. However, there was no significant difference in cell growth between SH1 and DW4/3-1.

Table. 2.1. Cell growth of SH1 and DW4/3-1 at different temperatures.

	32°C t_0 (cells/ml)	32°C t_{48hrs} (cells/ml)	35°C t_0 (cells/ml)	35°C t_{48hrs} (cells/ml)	37°C t_0 (cells/ml)	37°C t_{48hrs} (cells/ml)	37°C t_{56hrs} (cells/ml)
DW4-/3-1	1.0×10^6	1.2×10^8	1.0×10^6	1.5×10^8	1.0×10^6	3.0×10^7	1.1×10^8
SH1	1.2×10^6	1.6×10^8	1.2×10^6	1.7×10^8	1.2×10^6	3.4×10^7	1.5×10^8

2.6.3. The ability of SH1 to form fruiting bodies

The ability of fruiting body formation of SH1 and DW4/3-1 was tested on water-agar plates without any antibiotic. The plates were then incubated at 30°C, 32°C, 35°C, 37°C, and 39°C, respectively.

Both the mutant strain SH1 and the wild-type strain DW4/3-1 were able to develop even under heat shock conditions. At 30°C, 32°C, and 35°C, SH1 and DW4/3-1 cells started to aggregate about nine hours after the beginning of starvation. A Stalk was formed about 13 to

14 hours after the beginning of development. Both strains formed mature fruiting bodies after about 24 hours. The time dependent phenotype during the developmental cycle of SH1 was found to be the same as that described by G. T. Qualls et al. (Qualls *et al.*, 1978).

At 37°C and 39°C, SH1 and DW4/3-1 cells started to aggregate about 12 hours after the beginning of starvation. After about 20 hours, early stalk-like structures were formed by both strains. Then the developmental cycle stopped.

No difference was observed with cells of SH1 and DW4/3-1 (some results see Fig. 2.24, Fig. 2.25).

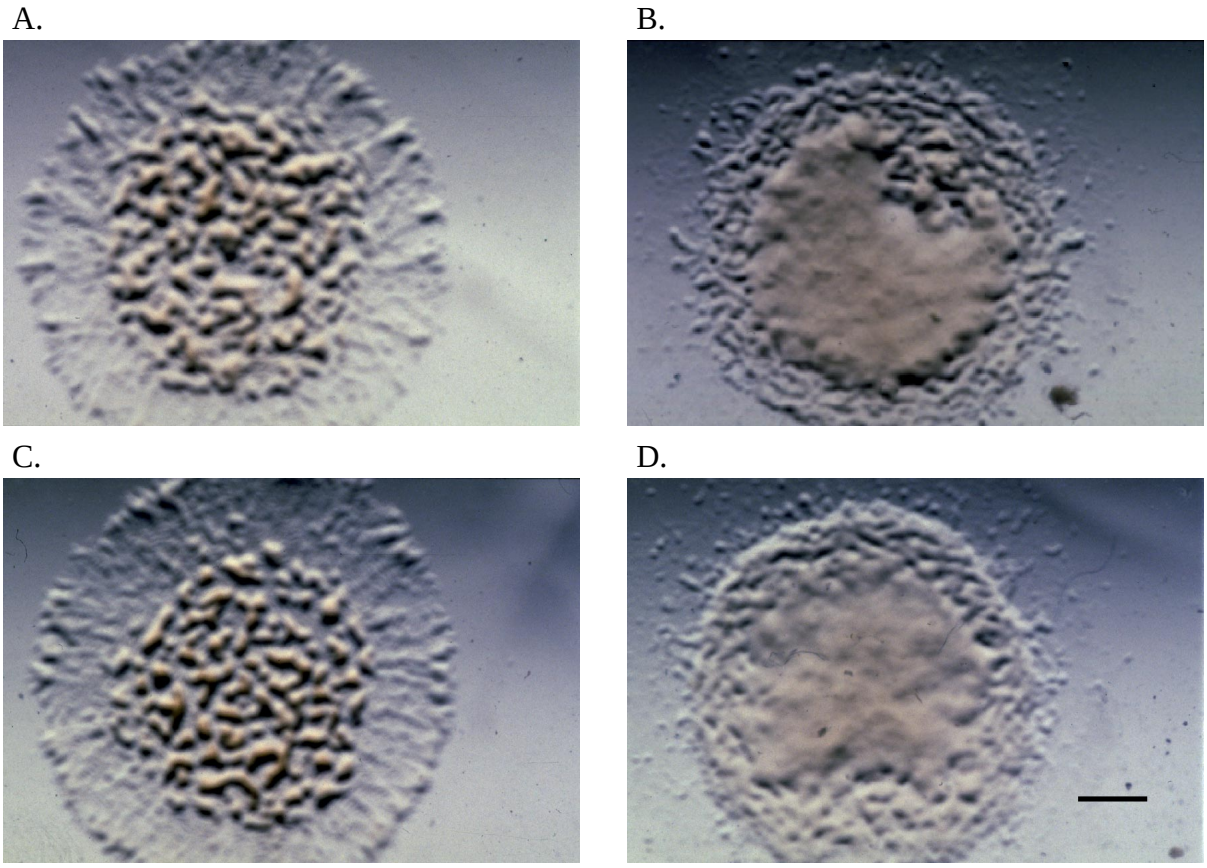


Fig. 2.24. Aggregation of SH1 and DW4/3-1 on water-agar, after 10 hours of development. A. SH1 at 32°C; B. SH1 at 37°C; C. DW4/3-1 at 32°C; D. DW4/3-1 at 37°C. The bar represents 1mm.

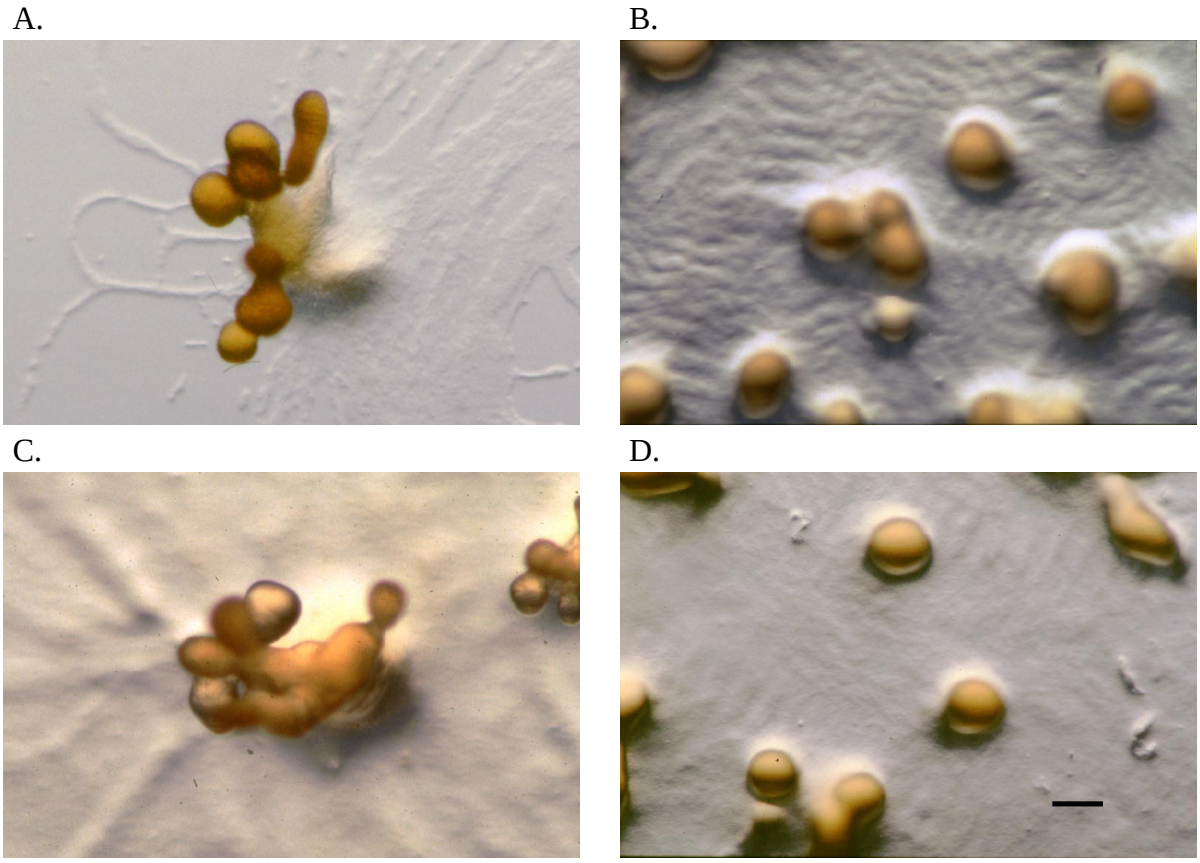


Fig. 2.25. Fruiting body formation of SH1 and DW4/3-1, after 24 hours of development. A. SH1 at 32°C; B. SH1 at 37°C; C. DW4/3-1 at 32°C; D. DW4/3-1 at 37°C. The bar represents either 20 μm in (A) and (C) or 50 μm in (B) and (D).

2.6.4. The ability of SH1 myxospores to germinate

The capability of *S. aurantiaca* myxospores to germinate was measured only at 32°C using the myxospores from fruiting bodies of SH1 and DW4/3-1 formed at 32°C. Swarming cells of SH1 or DW4/3-1 were visible after two days of incubation of the myxospores on Trypton agar. This result indicates that the myxospores formed by SH1 germinate as well as the DW4/3-1 myxospores.

2.6.5. The ultrastructure of SH1 myxospores

The ultrastructure of SH1 myxospores was examined in a cooperation with H. Lünsdorf (GBF, Germany). Immunoelectron microscopy was performed with the fruiting body derived myxospores of SH1 and DW4/3-1. HspA could be detected by protein A-gold conjugated anti-HspA-sera. HspA was not detected in the myxospores formed by SH1 (Fig. 2.26). This supports the Northern and Western analyses of SH1. As mentioned previously, HspA was present in DW4/3-1 myxospores. There was no difference between the SH1 myxospores and

that of DW4/3-1 in spore coat, in inclusion bodies, and in other intracellular structures of myxospores as examined by conventional electron microscopy (Fig. 2.27).

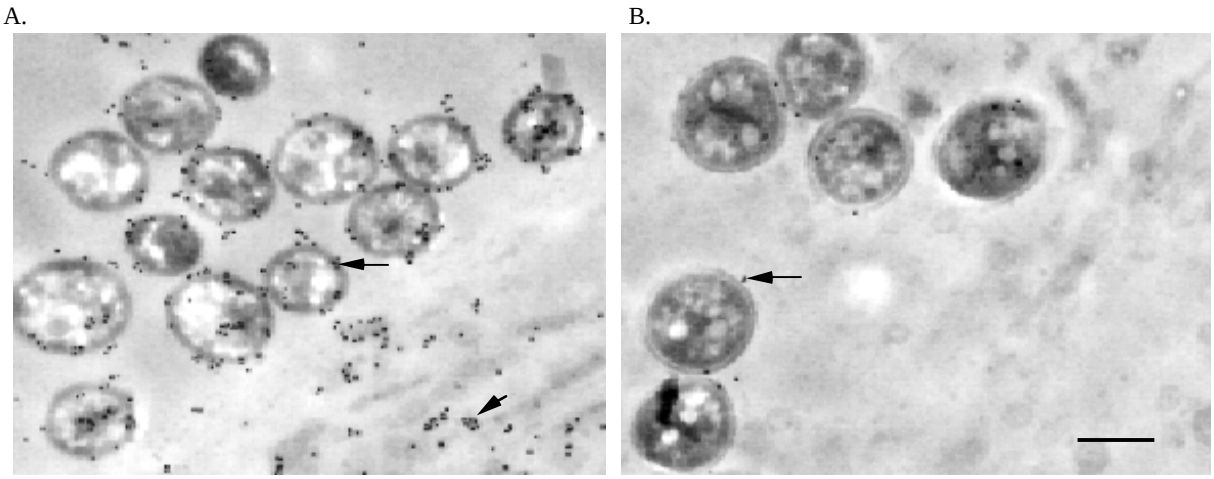


Fig. 2.26. Immunocytochemical detection of HspA polypeptide in the fruiting body of *S. aurantiaca*. A. DW4/3-1 fruiting body. The HspA polypeptide is indicated by arrows. B. SH1 fruiting body. The arrow shows a background signal. The bar represents 1.1 μm.

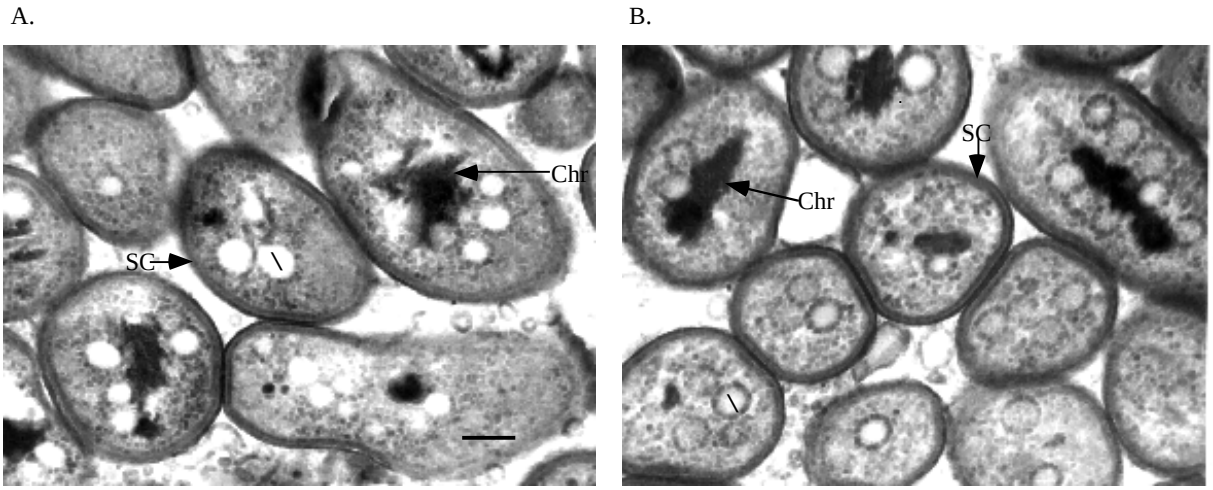


Fig. 2.27. The ultrastructure of myxospores within a *S. aurantiaca* sporangiole formed by DW4/3-1 (A) and SH1 (B). Chr, SC, and \ correspond to the bacterial chromosome, the spore coat and the inclusion body, respectively. The bar represents 250 nm.

With the exception of HspA synthesis, no phenotype difference was detected in vegetative cells or spores between strains DW4/3-1 and SH1 (summarised in Table 2.2). The physiologic function of HspA remains to be determined.

Table. 2.2. Phenotypic characteristics of SH1

Assays		*S. aurantiaca*	
		SH1	DW4/3-1
hspA gene (Southern analysis)		-	+
HspA protein (Western analysis)		-	+
Vegetative cell growth		normal	normal
Developmental cell cycle	Aggregation 30°C-39°C	+	+
	Stalk formation 30°C-35°C 37°C-39°C	+ partially formed	+ partially formed
	Fruiting body formation 30°C-35°C 37°C-39°C	+ -	+ -
	Sporulation 30°C-35°C	+	+
	Germination 32°C	+	+
Spore ultrastructure	Spore coat Inclusion body others	normal	normal

2.7. Investigation of the chaperone function of HspA *in vitro*

Since some small heat shock proteins have been proven to have chaperone properties, the similar assays were thus performed with HspA to elucidate whether HspA can act as a molecular chaperone.

2.7.1. Heterologous expression of *hspA*

In order to get enough HspA to perform the chaperone assays, *hspA* was expressed in *E. coli* using the expression vector pQE9 (Qiagen). The PCR product of the *hspA* gene (EMBL/GeneBank: M94510, bp 568-bp 1134) (see 2.1.1) was cloned into the *Sal*I/*Hin*dIII sites of pQE9 resulting in plasmid pSH11. In pSH11, the 5'-end of the *hspA* coding sequence

was fused to a DNA sequence encoding the enterokinase recognition site (DDDDK) harbouring DNA for 6 histidines at its 5'-end. The hybrid gene encodes a fusion protein (Fig. 2.28A). The expression of *hspA* is under the control of the T5 promoter that is fused to the *lac* operon. The generated fusion protein is named $HspA_{His}$ (Fig. 2.28B).

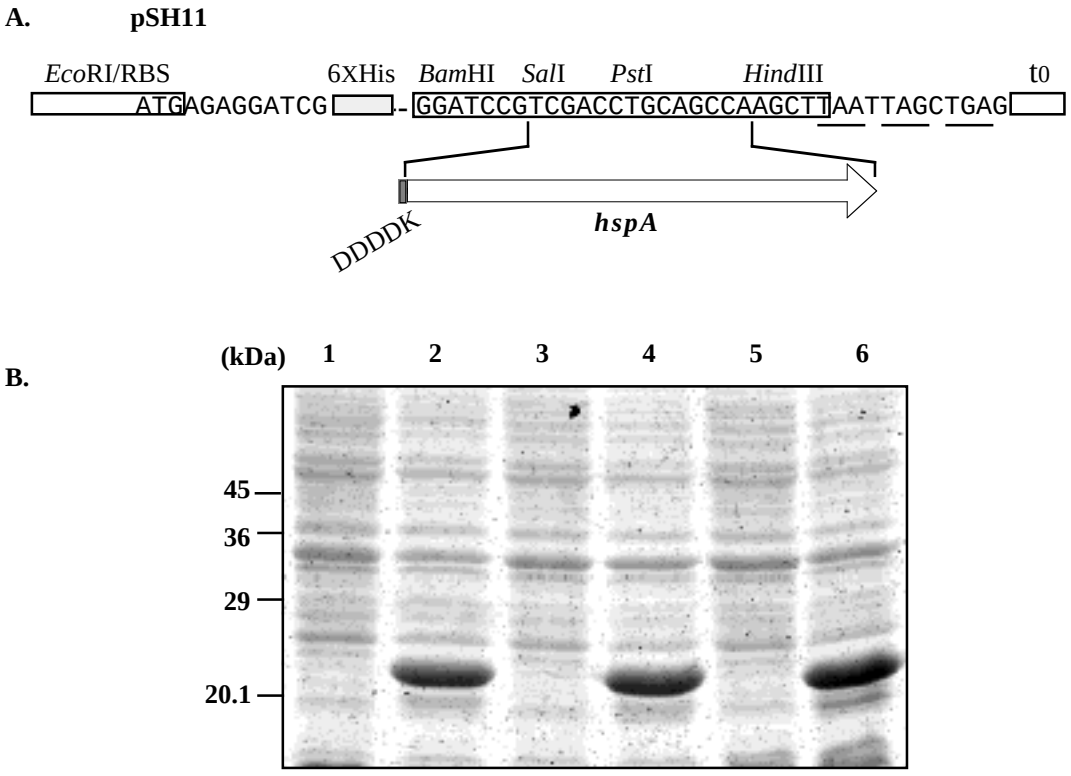


Fig. 2.28. Expression of *hspA* in *E. coli*. A. Schema of pSH11. Only the cloning sites are indicated. B. The synthesis of $HspA_{His}$ in *E. coli* M15 (Qiagen) was induced by 1 mM IPTG at 37°C for 3 hours (lanes 2, 4, and 6). Cell lysates before induction are shown in lanes 1, 3, and 5.

2.7.2. Purification of the recombinant HspA polypeptide under denaturing conditions

Plasmid pSH11 was transferred into the *E. coli* strain M15 (Qiagen) that contains a helper plasmid pREP4, which encodes the *lac* repressor. The expression of the hybrid gene depends on the addition of IPTG to induce the *lacZ* promoter. Induction of the hybrid gene by IPTG resulted mainly in an insoluble form of the fusion protein. This insoluble fusion protein was purified under denaturing conditions on a Ni-NTA agarose column (Fig. 2.29). This product of $HspA_{His}$ was used to generate anti-HspA-sera.

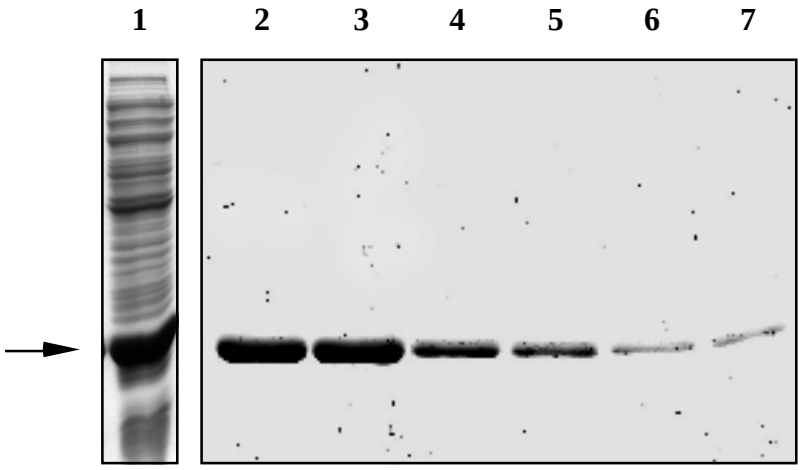


Fig. 2.29. Purification of $HspA_{His}$ on a Ni-NTA agarose column under denaturing conditions. Fractions (lanes 2-7) were visualised by Coomassie blue staining after SDS-PAGE on a 12% gel. Lane 1 shows the cell lysates. The arrow corresponds to $HspA_{His}$.

2.7.3. Synthesis and purification of soluble $HspA_{His}$

pSH11 was transferred into the *E. coli* strain GI698 (Invitrogen). As in *E. coli* M15 (2.7.1), the expression of *hspA* was regulated by the *lac* repressor. The difference is that the *lac* repressor gene was integrated in the genome of GI698. The expression of *hspA* was induced by IPTG at room temperature. Under this condition, more than 50% of the fusion protein was soluble (Fig. 2.30). The soluble recombinant $HspA_{His}$ was purified on a Ni-NTA agarose column without addition of any chaotropic reagent. Such native $HspA_{His}$ is suitable to perform biochemical assays.

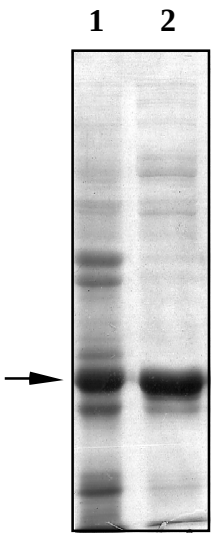


Fig. 2.30. Expression of *hspA* in *E. coli* GI698 at room temperature. The insoluble and the soluble fractions of the cell lysates were separated by centrifugation at 40,000 × g for one hour. The pellet (lane 1) and soluble fractions (lane 2) of cell lysate were separated by SDS-PAGE on a 12% gel. The arrow corresponds to $HspA_{His}$.

2.7.4. Determination of the molecular mass of $HspA_{His}$ monomer

In order to analyse the quaternary structure of $HspA_{His}$, it is important to determine the molecular mass of the monomer form of $HspA_{His}$. Mass spectrophotometry was performed with the native $HspA_{His}$ in a cooperation with R. Frank (ZMBH, Germany). The data obtained indicated the molecular mass of $HspA_{His}$ to be 21563.0 Da (Appendix 1). It agrees quite well with the calculated molecular mass of $HspA_{His}$ (21558.9 Da) with respect to the corrected ORF of *hspA.*

2.7.5. Elucidation of the oligomeric structure of $HspA_{His}$

An important characteristics of small heat shock proteins is their ability to form oligomers. It has been suggested that the oligomers are the functional units for small heat shock protein-dependent chaperone catalysis. To investigate whether $HspA_{His}$ assembles into a large oligomer, HPLC size exclusion chromatography was performed with the native recombinant $HspA_{His}$. The elution profile (Fig. 2.31) showed one predominant peak at about 560 kDa, which corresponds to a complex composed of 26 subunits of $HspA_{His}$. 10-fold dilution did not change the elution peak of $HspA_{His}$ (data not shown). This suggests that the oligomeric structure of $HspA_{His}$ is stable.

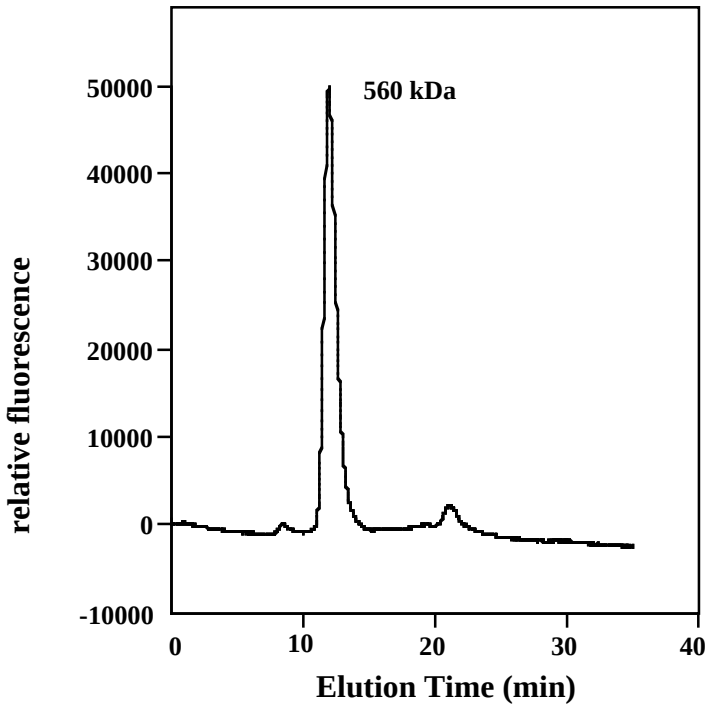


Fig. 2.31. Detection of the $HspA_{His}$ oligomeric complex by HPLC size exclusion chromatography (SEC). SEC was performed with 10 µg $HspA_{His}$ in 100 µl of 40 mM HEPES buffer (pH 7.5) at 25 °C, using a TosoHaas TSK G4000 SW column.

2.7.6. Analyses of the chaperone activities of $HspA_{His}$

Citrate synthase (CS) and insulin have often been used as model substrates to study the influence of small heat shock proteins on protein folding and unfolding reactions (Farahbakhsh *et al*., 1995; Jakob *et al*., 1993).

2.7.6.1. The interaction of $HspA_{His}$ with chemically denatured CS

To test whether $HspA_{His}$ can interact with an unfolded protein and facilitate protein folding, the effect of $HspA_{His}$ on folding of CS was studied. The mitochondrial CS, from pig heart (Boehringer, Mannheim), is a homodimer with a molecular mass of 49 kDa.

15 μM CS (dimer) was denatured in 6 M guanidine hydrochloride (GuHCl) buffer at 25 °C for two hours. After a 1:100 dilution in 40 mM HEPES (pH 7.5) buffer, the refolding procedure of denatured CS was initiated (Fig. 2.32). When chaperone is absent in the dilution buffer, CS aggregates immediately as shown by an increase of light scattering. Aggregation is due to an incorrect folding.

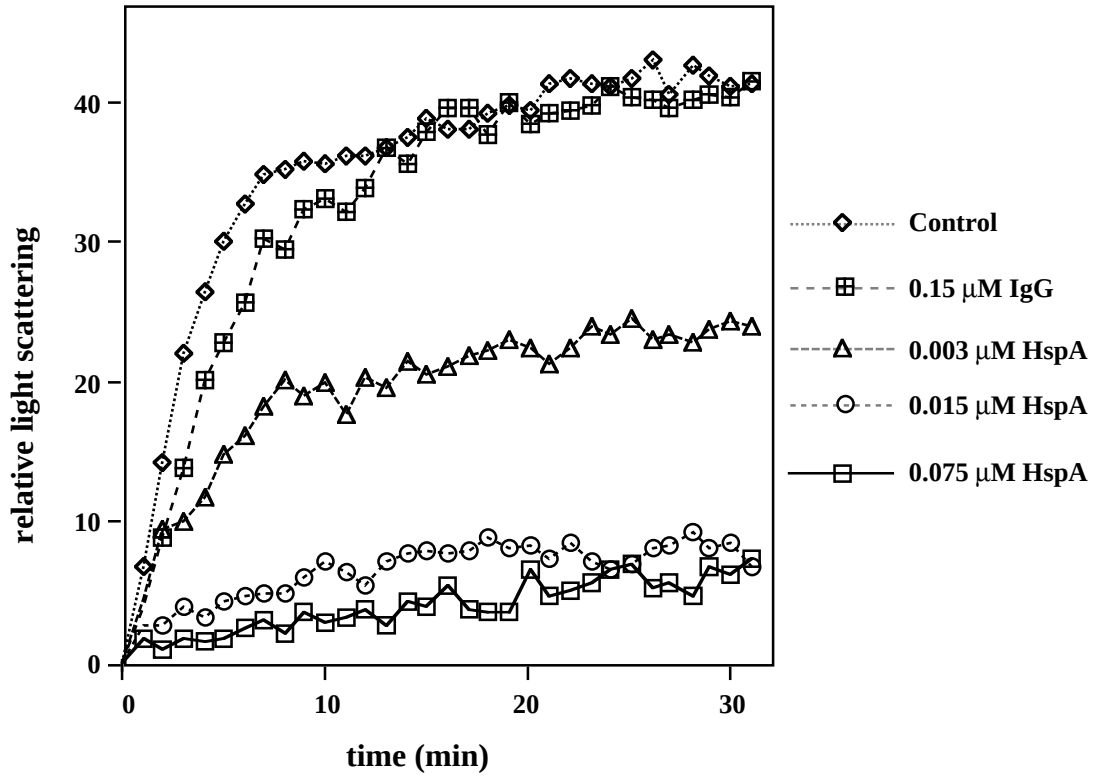


Fig. 2.32. Aggregation of chemically denatured CS in the absence and presence of $HspA_{His}$. The kinetics of aggregation was determined by light scattering. Guanidine hydrochloride (GuHCl) denatured CS was diluted to a final concentration of 0.03 μM (monomer). The concentrations of $HspA_{His}$ and bovine IgG are indicated in the graph.

The presence of $HspA_{His}$ in the dilution buffer suppressed spontaneous aggregation of CS. Increasing amounts of $HspA_{His}$ reduced the degree of CS aggregation. The addition of 0.015 μM $HspA_{His}$ (oligomer) in the dilution buffer suppressed the spontaneous aggregation of CS almost completely. Bovine IgG was used as a control protein in this assay, which had no effect on the refolding of CS. Two known small heat shock proteins were tested also in this aggregation assay. One is α-crystallin from bovine eyes, and the other is Hsp25 from mouse (kindly provided by M. Ehrnsperger, University of Regensburg, Germany). Both proteins did not prevent the aggregation of the chemically denatured CS (data not shown).

2.7.6.2. The influence of $HspA_{His}$ on the chemically induced aggregation of insulin

Insulin was selected to investigate whether $HspA_{His}$ can prevent aggregation during its unfolding. Insulin, from bovine pancreas (Sigma), is composed of A-chain and B-chain that are joined by two interchain disulphide bonds. The A-chain contains an extra intrachain disulphide bond. The molecular mass of insulin is about 5.8 kDa, and that of the B-chain is 3.3 kDa. Reduction of the insulin interchain disulphide bonds leads to aggregation and precipitation of the B chain while the A chain remains in solution (Farahbakhsh *et al*., 1995).

Fig. 2.33 shows that the B-chain of insulin underwent spontaneous aggregation after addition of dithiothreitol (DTT) to a final concentration of 20 mM. When mouse Hsp25 was present in the buffer before the addition of DTT, the spontaneous aggregation of the B-chain was suppressed. However, $HspA_{His}$ did not prevent the aggregation of the B chain of insulin.

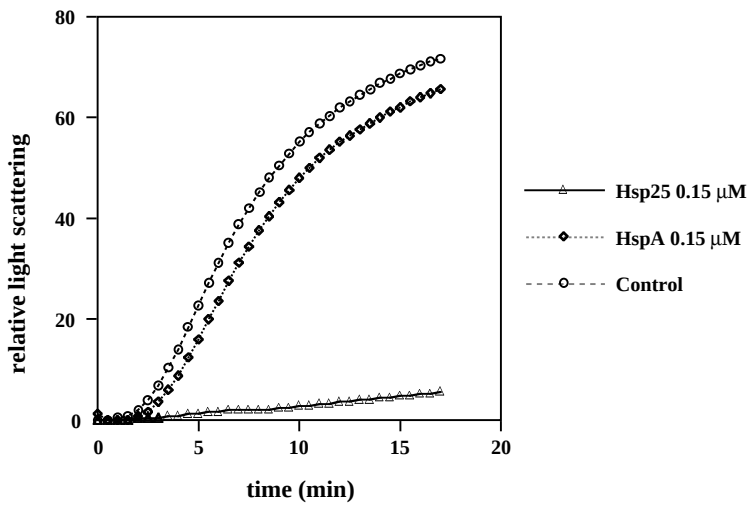


Fig. 2.33. Aggregation of the B chain of insulin after addition of dithiothreitol (DTT) to a final concentration of 20 mM in the absence or presence of HspAHis or Hsp25. The kinetics of aggregation was determined by measuring light scattering of the samples. Insulin was diluted to a final concentration of 0.25 mg/ml. The concentrations of HspAHis and Hsp25 used are indicated in the graph.

2.7.6.3. The effect of HspA on the reactivation of chemically denatured CS

To understand how $HspA_{His}$ interacts with denatured CS, the recovery of the enzymatic activity of unfolded CS was determined.

Under the chosen conditions, when chemically denatured CS was diluted in the dilution buffer, the specific activity of CS recovered spontaneously up to about 20% of that of native CS in the absence of chaperone. Surprisingly, in the presence of $HspA_{His}$ in the dilution buffer, spontaneous reactivation of CS was completely blocked (Fig. 2.34). This may be due to a stable complex formed between unfolded CS and $HspA_{His}$. Bovine IgG did not affect spontaneous reactivation of unfolded CS (data not shown).

The chaperone function of small heat shock proteins needs a cooperation with a cofactor or other chaperones (Ehrnsperger *et al.*, 1997; Veinger *et al.*, 1998). Therefore, it was tested whether such factors might help to dissociate the $HspA_{His}$-CS complex. It had been reported that Hsp70 or oxaloacetic acid (OAA), a substrate and ligand of CS, could trigger the dissociation of the complex formed by the thermally denatured CS and mouse Hsp25. The same approach was used to test whether these factors might facilitate the reactivation of $HspA_{His}$ bound CS.

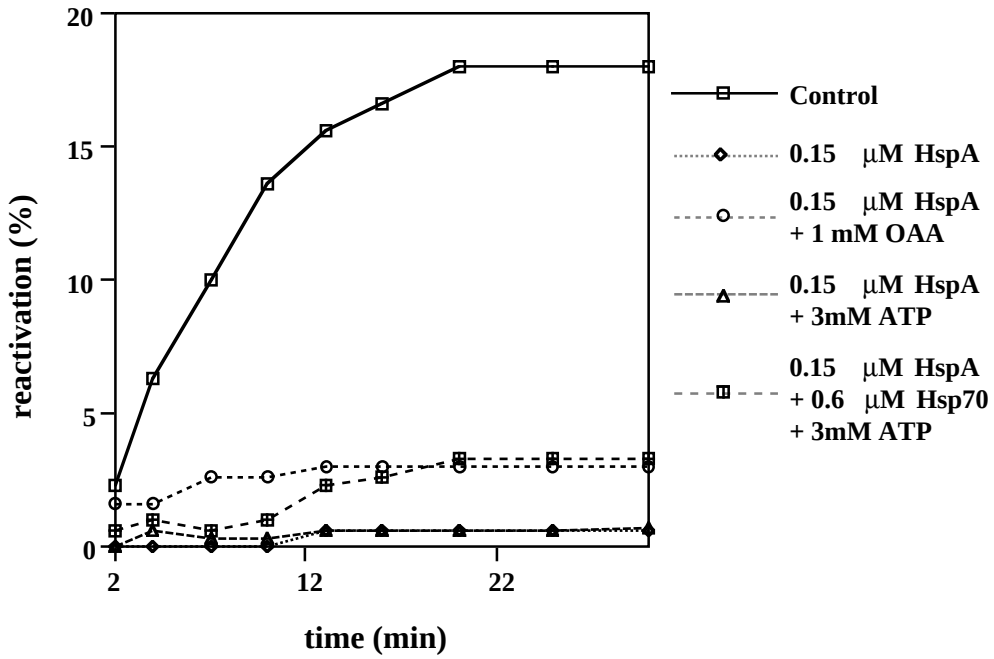


Fig. 2.34. Reactivation of unfolded CS in the absence and presence of $HspA_{His}$. Unfolded CS was diluted in a buffer without $HspA_{His}$ or with 0.15 μM $HspA_{His}$ to a final concentration of 0.15 μM. 30 minutes after dilution of denatured CS, Hsp70 or OAA or ATP was added to the reaction, individually or simultaneously as indicated in the graph.

After incubation of $HspA_{His}$ with unfolded CS for 30 minutes, Hsp70 from yeast (kindly provided by M. Ehrnsperger, University of Regensburg, Germany) or OAA was added to the dilution buffer. As shown in Fig. 2.34, neither Hsp70 nor OAA led to the dissociation of bound CS from $HspA_{His}$. As measured by its activation, CS activity detected was slightly higher than zero. This indicates that ATP had no effect on stimulation of CS reactivation in cooperation with Hsp70.

2.8. Conclusions on the function of HspA

HspA is an abundant small heat shock protein in *S. aurantiaca* cells, which is mainly located along the cell wall of heat shocked cells or fruiting body derived myxospores. However, thermotolerance and differentiation of *S. aurantiaca* cells are not affected when this protein is absent.

The His-tagged HspA protein tends to assemble into a large complex that consists of 26 subunits with a molecular mass of 560 kDa as judged by SEC. This oligomer of $HspA_{His}$ is able to interact with unfolded CS and prevents its precipitation from solution. But it has no effect on the precipitation of unfolded B-chain of insulin. A stable complex may be formed between $HspA_{His}$ and unfolded CS since CS activity was not recovered.

III. Discussion

S. aurantiaca is an important model organism to study development. The research on gene regulation with this bacterium just started. *hspA* is the first gene that was studied at the transcriptional regulation level in *S. aurantiaca*. The knowledge about sigma factors and signal transduction in this bacterium is still poor. This makes it very difficult to understand the transcriptional regulation of *hspA*.

The lack of efficient methods of bacterial genetics impedes strongly the genetic research of *S. aurantiaca*. Although, very little is known about the putative *attB* site of DW4/3-1, the *attP-attB* recomination was successfully used for the first time in *S. aurantiaca* to analysis the *hspA* promoter activity in this work.

In many cases, the physiological function of a certain protein can be elucidated by inactivation of its encoding gene. However, no detectable change in phenotype was observed as a consequence of *hspA* deletion. Considering the properties of small heat shock proteins, the function of HspA was thus characterised by *in vitro* biochemical assays.

3.1. Transcriptional regulation of *hspA*

3.1.1. Transfer of foreign DNA into *S. aurantiaca*

Up to now, no plasmid has been found that replicates in *S. aurantiaca*. Until recently, the only way to transfer foreign DNA into *S. aurantiaca* was by conjugation from *E. coli* to *S. aurantiaca* (Glomp *et al*., 1988; Silakowski *et al*., 1996). The conjugation system consists of two components: special *E. coli* donor strains (e.g. *E. coli* S17-1) that carry the transfer genes of the IncP-type plasmid RP4 in their chromosome, and derivatives of vector plasmids (e.g. pSUP102) that contain the RP4-specific Mob-site (Simon *et al*., 1986). To introduce foreign DNA into *S. aurantiaca*, this foreign DNA must be first cloned into such a vector and then transferred into *E. coli* donor cells. After mating *E. coli* donor cells with *S. aurantiaca* recipient cells, the plasmid that carries the foreign DNA is transferred to the *S. aurantiaca* cells and integrated into the genome of *S. aurantiaca via* homologous recombination.

At the beginning of the promoter analysis in course of this work, it has been tried to use the RP4-mediated conjugation system to introduce the promoter region of *hspA* into the genome of *S. aurantiaca*. Therefore the *rtrpA-lacZ* reporter gene and Tn5 derived *neo* gene cassette was cloned into a derivative of plasmid pSUP102 to generate pSH3 (2.3.1.1). However, this conjugation system has disadvantages. First, the recombination effiency with this system is low. Second, the size of pSUP102 is about 6 kbp and its copy number in *E. coli*

cells is low because it carries the replication origin of plasmid p15A. Though part of the pSUP102 sequence was deleted, handling of the derivatives of this plasmid was inconvenient.

After adoption of electroporation to *S. aurantiaca* (Stamm *et al*., 1999), this method was used to transfer foreign DNA into this organism. In comparison to the conjugation system, the electroporation method gives obviously more transformants. Since then the plasmids used in the promoter assays of this work were constructed using the vector pBluescript SK- (Stratagene).

3.1.2. The *attP-attB* integration system

Mx8 is a temperate myxophage that infects *M. xanthus* (Martin *et al*., 1978). Integration of Mx8 into the host chromosome requires the product of *trans*-acting integrase gene (*int*) and the *cis*-acting *attP* sequence (Magrini *et al*., 1997; Salmi *et al*., 1998). A plasmid that carries the *int* gene and the *attP* site of Mx8 can recombine efficiently with the bacterial *attB* locus and form a stable co-integrate with the genome of *M. xanthus*. Such a plasmid is used as a cloning vector to introduce foreign DNA into the *M. xanthus attB* site for Mx8 (Li and Shimkets, 1988).

The *attP* site mediated site-specific integration in *M. xanthus* permits a foreign DNA to insert into the 3'-ends of either of two tandem tRNA-Asp genes: *trnD1* and *trnD2* located in the *attB* loci of *M. xanthus* genome (Magrini *et al*., 1999). Moreover, the *attP* × *attB1* (within *trnD1*) reaction is highly favoured.

For *S. aurantiaca*, it is certainly interesting to know whether Mx8 can be used as a tool to introduce foreign DNA into this organism efficiently and ectopically. If the answer would be yes, the genetic manipulation of *S. aurantiaca* would be easier. For this purpose, the Mx8 *attP* site and *int* gene containing plasmid pSH8 was introduced into *S. aurantiaca* DW4/3-1. The result proved that the Mx8 *attP* site could integrate efficiently into the genome of *S. aurantiaca*. Southern analysis was performed thereafter with the genomic DNA of the recombinants. It revealed a unique *attB* site in the genome of *S. aurantiaca*. But, this does not exclude the possibility that there are two *attB* sites that are located closely in the *attB* locus of *S. aurantiaca* genome and are not distinguishable by Southern hybridisation as in the case of *M. xanthus* (2.3.1).

Before the promoter mapping assays of *hspA* were performed, it was very important to test whether P*hspA* integrated in the *attB* site has the same activity as in its original locus (2.3.2). This consideration is mainly due to the observations with the similar work in *M. xanthus*. The strength of some C signal dependent promoters of *M. xanthus* is reduced

after integration into the *attB* site, as compared with the promoter strength at their original sites (Brandner and Kroos, 1998; Fisseha *et al*., 1996). Expression of the hybrid gene obtained by fusion of the indicator gene `r`*trpA-lacZ* to P*hspA* was thus studied after integration either into the *hspA* locus or into the *attB* site. Moreover, for each *attP-attB* mediated integration, ß–galactosidase activity was measured at least with three independent strains. No significant difference of the maximum ß-galactosidase activity was observed with independent transformants obtained from a single transformation. The maximum expression of *hspA* as measured by ß-galactosidase activity was not altered significantly when the P*hspA–lacZ* construct was inserted into *hspA* locus or into *attB* site. The data show that the *attP-attB* system can be used for the P*hspA* analyses.

Despite of the success with P*hspA* study using *attP-attB* recombination, whether this ectopic recombination system can be generally used in *S. aurantiaca* remains to be confirmed. It was observed that the ability to form fruiting body is affected in some *attP-attB* recombinants though the synthesis of ß-galactosidase was not impaired (data not shown). The reason for this phenotype is not known.

3.1.3. The transcription of *hspA* under heat shock conditions

Northern analysis revealed two transcripts of *hspA* to be formed during heat shock, whereas only one transcript was detected after indole treatment. Two possible reasons are the transcription of *hspA* to start or to terminate at two different sites under heat shock conditions, while only at one site during indole treatment. The size of the two *hspA* transcripts differs by only 50 nts. The two transcriptional initiation sites, if they would exist, should be detected in the primer extension assay when using the same primer. However, only one identical reverse transcript was detected by primer extension either using total RNA from heat shocked DW4/3-1 cells or using that from indole treated cells as template.

In the promoter mutagenesis assays, deletion in the promoter region revealed that removal of the region from bp -35 to bp -6 relative to the transcription start site of *hspA* completely elinimated the expression of *lacZ* reporter gene. If there would be a further transcription start site located 50 bp upstream of the identified transcription start site, the reporter gene would be expressed under the control of the mutated P*hspA*. Furthermore, reverse transcription PCR (RT-PCR) experiments were performed (data not shown). The results showed that the sequences downstream of the *hspA* termination site could be amplified using total RNA from heat shocked *S. aurantiaca* cells as template. These results negate the possibility that the two transcripts of *hspA* are the result of two transcription start sites. But, it

is still unclear whether the two transcripts are the result of two transcription termination sites of *hspA* or the result of other unknown modifications of *hspA* mRNA under heat shock conditions. The transcription termination site of *hspA* should be characterised by S1 mapping assay of the 3'-end of the *hspA* mRNA.

3.1.4. The sigma factor for *hspA* transcription

The eubacterial sigma factors are divided into two main families: the σ^{70} and the σ^{54} family. The σ^{70} family is composed of several structurally and functionally related subgroups, while the σ^{54} family contains only one group of sigma factors. Most sigma factors belonging to the σ^{70} family recognise consensus sequences in two regions centred bp -35 and bp -10 relative to the transcription start site, whereas the members of the σ^{54} family recognise a consensus sequence centred bp -24 and bp -12.

The consensus sequences recognised by different sigma factors have been summarised by M. M. S. M. Wösten (Wösten, 1998) (Table 3.1).

The heat shock genes of bacteria are transcribed by RNA polymerase bearing either a σ^{32} (e.g., in *E. coli* and α-,γ-subgroup of proteobacteria), a σ^{E} (e.g., in *E. coli*), a σ^{B} (e.g., in *B. subtilis*) or a σ^{70} (e.g., in *E. coli* and in *B. subtilis*).

Comparison of P*hspA* with the consensus sequences of the known sigma factors indicated that P*hspA* is neither a typical σ^{32} dependent promoter nor a typical σ^{70} or a σ^{54} dependent promoter (Table 3.1). Only the 5'-CTTG-3' motif that located in the -35 region of P*hspA* is similar to the -35 consensus sequence of σ^{32} dependent promoters. But the -10 region of P*hspA* completely differs from that of σ^{32} dependent ones.

It is possible that a species-specific sigma factor is employed to recognise P*hspA*. σ^{B} is induced by heat shock in S. *aurantiaca* (Silakowski *et al*., unpublished) and thus is a candidate for the transcription of heat shock genes. However, the expression of *hspA* in a σ^{B} mutant of *S. aurantiaca* as detected by Northern (2.3.4) and Western (data not shown) analyses indicated that *hspA* is independent on the RNA polymerase containing σ^{B}. Whether *hspA* is transcribed by RNA polymerase containing σ^{A} (the house keeping sigma factor of *S. aurantiaca*), or an alternative sigma factor remains unknown.

Deletion analyses of P*hspA* suggest that the region ranging from bp -35 to bp -6 upstream of the *hspA* transcription start site carries the recognition site for the RNA polymerase. The insertion of a *Cla*I recognition hexanucleotide sequence between bp -36 and bp -35 did not affect the *hspA* expression. This suggests that the -35 region of P*hspA* should be downstream of bp -35. To determine the sigma factor recognition site, point mutations have to be

introduced in the promoter region of *hspA* and also DNA footprinting analyses have to be performed.

Table 3.1. Consensus sequences recognised by various eubacteria sigma factors

σ^{70} family	Name	Consensus sequence			Reference
		-35	spacer	-10	
Primary σ-factors	σ^{70}, RpoD, SigA	TTGACA	16-18	TATAAT	
Stationary-phase σ-factors	σ^{38}, RpoS			CTATACT	
Flagella σ-factors	σ^{28}, FliA, SigD	TAAA	15	GCCGATAA	
ECF σ-factors	σ^{E}, SigE	GAACTT	16-17	TCTRA	
Heat shock σ-factors	σ^{32}, RpoH	CTTGAAA	11-16	CCCATnT	
	σ^{B}, SigB	GTTTAA	12-14	GGGTAT	
Sporulation σ-factors	σ^{H}, SpoOH	AGGAWWT	12-14	RGAAT	
	σ^{F}, SopoIIAC	WGCATA	14-15	GGnRAYAMTW	
	σ^{E}, SpoIIGB	GKCATATT	13-15	CATACAMT	
	σ^{G}, SpoIIIG	TGAATA	17-18	CATACTA	
	σ^{K}, SpoIIIC	AC	16-17	CATAnAnTA	
σ^{54} family		-24	Spacer	-12	
	σ^{N}, RpoN, SigL	TGGCAC	5	TTGCW	
hspA promoter		-35	Spacer	-10	
		CTTGCGC	16	TACGGT	This work

Ambiguous codes: N, any base; R, A or G; W, A or T; Y, C or T; M, A or C; K, G or T. Most data from M. M. S. M. Wösten (Wösten, 1998) and the references cited therein if not indicated.

3.1.5. Comparison of P*hspA* with promoters of *M. xanthus*

hspA is induced not only by heat shock but also by development. Possibly, transcription of *hspA* is similarly regulated as that of certain developmental genes.

P*hspA* was compared with development dependent promoters of *M. xanthus* (Biran and Kroos, 1997; Brandner and Kroos, 1998; Downard *et al*., 1988; Fisseha *et al*., 1996; Gulati *et al*., 1995; Hanlon *et al*., 1997; Keseler and Kaiser, 1995; Li *et al*., 1992; Martinez-Argudo *et al*., 1998; Ogawa *et al*., 1996; Romeo and Zusman, 1991). It was found that P*hspA* has similarity to the promoter of the *fruA* gene (Ogawa *et al*., 1996). Fig. 3.1 shows that the hexanucleotide sequence of the -10 region of both promoters matches in five positions. The promoter sequences of the -35 region of both promoters are also quite similar.

FruA is a transcription factor involved in signal transduction downstream of the C-signal (Eiiehauge *et al.*, 1998). The expression of *fruA* starts after 6 hours of development and reaches the maximum level after 12 hours. *fruA* is not expressed during vegetative growth. The transcription of *fruA* depends on A- and E- signals, while C-signal may activate FruA post-translationally *via* a two-component regulatory system. Due to the similarity of their promoter sequences, the transcription of *hspA* may share some factors involved in the regulation of *fruA*.

```
                    -35                          -10          +1
fruA  5'-ATTGGGGCTGGTTCGCGTCTGCGCTTTCCCGGTGATAGGGTTTCTGCGTTGG-3'
hspA     5'-GCCATGCCGTCTTGCGCTGTCTCGAAGCTGTCTTACGGTCTGGATGGCGG-3'
```

Fig. 3.1. Alignment of the *hspA* and *fruA* promoter sequences. The transcription start site (+1), the -10, and the -35 regions of both promoters are indicated.

3.1.6. Activation of P*hspA*

The promoter region of *hspA* was identified by deletion mapping of the upstream region of *hspA*. This analysis revealed that different upstream regions of *hspA* are required for the maximum expression of *hspA* in response to heat shock and to indole treatment. Under both stress conditions, transcription of *hspA* starts at the same site. This suggests that *hspA* is transcribed by an RNA polymerase bearing an identical sigma factor under both conditions. To respond to different stress signals, different factors may participate in the activation of P*hspA*. In accordance with this assumption, the activation and strength of P*hspA* are different under both stress conditions. Induction of *hspA* by heat shock is much faster than that by indole treatment and the maximum level of *hspA* reached during heat shock is twice of that reached after the addition of indole (as measured by the determination of ß-galactosidase activity).

3.1.7. Transcriptional regulation of *hspA*

Gel shift experiments revealed that certain proteins from the cell extracts of DW4/3-1 cells band to the G3 region of P*hspA*. Binding is enhanced by heat stimulation. Northern analysis showed that there is no flanking gene(s) of *hspA* expressed under heat shock conditions using the insert of pSH1 as a probe (data not shown). It can thus be speculated that G3 acts as a regulatory element involved in the transcriptional initiation of *hspA*. Furthermore,

promoter deletion assays supported the assumption that G3 interacts with putative transcription activator(s).

In bacteria, two general models for transcription activation have been found (review see (Bowman and Kranz, 1998; Rhodius and Busby, 1998)). In the first model, the activators bind to the upstream region near the σ^{70} recognition and RNA polymerase binding sites, between bp -30 and bp -80 upstream of the transcription start site. The second model was first described for the σ^{54} dependent promoters (Merrick, 1993; North *et al.*, 1993) and late also found in a house keeping sigma factor dependent promoter of *Rhodobacter capsulatus* (Colado-Vides *et al*., 1991). In this model, the activators bind more than 100 bp upstream of the transcription start site. It is termed as a transcription enhancer. In the case of the *hspA* promoter, G3 ranges from bp -91 to bp -173 upstream of the *hspA* transcription start site. It resembles the transcription enhancer model, and suggests that the G3 region contains the putative enhancer binding site(s).

It is noteworthy that the deletion of the G3 region did not completely abolish the activity of P*hspA*. Deletion of the region including G3 and the region upstream of the -35 region of P*hspA* strongly impaired the activity of P*hspA*. Similar results were obtained by the promoter mapping assays. In strain SH2821, the 97 bp sequence upstream of the *hspA* translational start site was fused to *rtrpA-lacZ* gene. Though the 97 bp sequence harbours the RNA polymerase binding site, *hspA* was not expressed during heat shock. These results suggest that there must be another *cis*-acting element proximal to the -35 region of P*hspA*. It has been reported that many bacterial promoters contain an upstream element that is located just upstream of the -35 region ranging from -60 to -40 named UP element. This element contains two conserved regions, an 11 bp distal region (-57 to -47, AAA(a/t)(a/t)T(a/t)TTTT) and a 4 bp proximal region (-44 to -41, AAAA). It contacts with the α subunit carboxy-terminal domain of RNA polymerase and stimulates transcription of bacterial genes *in vivo*, as well as *in vitro* in the absence of protein factors other than RNA polymerase. The effects of the UP element correlate generally with their similarity to the consensus sequences (Estrem *et al*., 1998; Rhodius and Busby, 1998; Ross *et al*., 1998; Ross *et al*., 1993). In P*hspA*, no A+T rich region is located upstream of -35 region. As the genome of myxobacteria has a high G+C content, the overall promoter sequences of *hspA* has a relative low A+T content as compared to that of *E. coli* promoters. It was found that the RNA polymerase of *S. aurantiaca* bearing σ^{70} from *E. coli* could not initiate the *E. coli* gene transcription *in vivo* (personal communication with H. U. Schairer, ZMBH, Germany). This suggests that the RNA polymerase of *S. aurantiaca*

may recognise different promoter sequences. It is thus possible that the upstream sequences of the -35 region of P*hspA* functions as an UP element and interact with the carboxy-terminal domain of the α subunit of the *S. aurantiaca* RNA polymerase. Therefore, P*hspA* may contain three RNA polymerase recognition sequences: a -10 region, a -35 region, and an UP element.

The gel shift experiments revealed two retarded bands of labelled G2 or G3. It suggests that G3 carries more than one binding site for putative transcription enhancer(s). This was proven by deletion mutagenesis of P*hspA*. In strain SH2813, deletion of the first inverted repeat of G3 reduced the P*hspA* activity to about 55%. Deletion of the second inverted repeat of G3 (strain SH2811) resulted in the reduction of the P*hspA* activity to about 65%. Deletion of most of G3 (covering the both inverted repeat regions) reduced the P*hspA* strength further to 38% (strain SH2816). These results are in accordance with those observed by promoter mapping assays. In strain SH2819 that harbours 192 bp of the upstream sequence of *hspA* fused to r*trpA-lacZ*, about 50% of the P*hspA* activity is retained. These results suggest binding of the putative enhancer(s) to the different sites of G3 seems to be independent from each other.

Taken together, the transcription regulation of *hspA* is mainly positively controlled under heat shock conditions.

As stated in the Introduction, a repressor mediated transcriptional regulation of heat shock gene expression is widely present in eubacteria. The promoter sequences of *hspA* were compared with the regulatory elements of other known heat shock genes in bacteria. No similarity was found. No CIRCE, or ROSE, or IR element was detected downstream of the transcription initiation site of *hspA*. Furthermore, many proteobacteria, particularly the members of γ subgroup, have sigma factor homologous to RpoH protein that is functionally related to *E. coli* σ^{32}. However, P*hspA* shows no homology to the σ^{32}-dependent promoters. This suggests the transcription of *hspA* to be regulated by an alternative mechanism of heat shock response.

3.1.8. Phosphorylation and transcriptional regulation of *hspA*

The gel shift assays indicated that binding of the transcription regulator(s) to G2 as well as to G3 depends not only on heat shock but also on phosphorylation. This suggests the regulation of *hspA* expression to be governed by a signal transduction system.

In bacteria, numerous signal transduction systems are evolved to allow the cells to respond to sudden and adverse changes of environment. Such systems often employ transmembrane receptors that sense extracellular signals and transmit information to

intracellular signalling components that in turn regulate the target gene expression. Among these systems, the two-component signal transduction systems are ubiquitous in bacteria, which typically consist of a sensor histidine kinase and a response regulator (review see (Goudreau and Stock, 1998)). Stimulation of the sensor by a specific signal results in sensor dimerisation and phosphorylation of a histidine residue located near the kinase catalytic domain. This phosphate group is then transferred to an aspartic acid residue within the receiver domain of the response regulator. Phosphorylation of the response regulator, in turn, controls the effector function, which is usually, but not always, involved in transcriptional regulation of target genes.

Eukaryotic-like signalling pathways have been found in myxobacteria. In *M. xanthus* a large family of Ser /Thr protein kinases (Pkn) have been found (Hanlon *et al*., 1997; Zhang *et al*., 1996). Among them, Pkn2, Pkn6 and Pkn9 are transmembrane kinases, while Pkn1 and Pkn5 are cytoplasmic protein kinases. Such kinases as well as a tyrosine kinase (Frasch and Dworkin, 1996) are demonstrated to be involved in the development of *M. xanthus*.

In *S. aurantiaca*, it has been found that the synthesis and degradation of inositol phospholipids are stimulated during Ca^{2+}-induced cohesion (Benaissa *et al*., 1994). In addition, a phospholipase C activity increases during this process and is stimulated by the presence of GTPγS, an inhibitor of G protein cycling. Therefore, it is proposed that a G–protein-like pathway exists in *S. aurantiaca*. Such a signalling pathway may be also involved in the regulation of *hspA*.

3.2. Function of the HspA protein

3.2.1. Expression of *hspA* in *E. coli*

Using the Qiagen expression system (Qiaexpresss kit), *hspA* gene was first cloned into the expression vector pQE9 (pSH11) and transformed into *E. coli* strain M15. However, most of the fusion protein formed was insoluble and was found in inclusion bodies, which were detected by immunoelectron microscopy (cooperation with H. Lünsdorf, GBF, Germany, data not shown). This fusion protein was purified under denaturing conditions. Efforts were made to get soluble fusion protein by removal of urea from the protein preparation by stepwise dialysis (urea concentration in the dialysis buffer is reduced from 6 M to 0 M, gradually). However, the fusion protein precipitated during dialysis when the concentration of urea was lower than 3 M (data not shown). To get a soluble fusion protein preparation, a strain from an expression system of Invitrogen (ThioFusion™ Expression kit) was employed and the *hspA*

carried by pSH11 was expressed in *E. coli* strain GI698 at room temperature. More than half of the fusion protein formed under this condition was soluble. The possible reason for the improved solubility is the slow induction of *hspA* expression at lower temperature. It does not exclude that the host strain GI698 has a genetic background that somehow facilitates the fusion protein to fold correctly. However, the manufacturer did not offer such information (personal communication with M. Younessian, Invitrogen). The soluble fusion protein was purified under native conditions. Theoretically, such soluble fusion protein should be folded in a correct way and thus can be used for biochemical assays.

3.2.2. The quaternary structure of $HspA_{His}$

$HspA_{His}$ has an additional His-tag with 6 Histidine residues and an enterokinase recognition site at the N-terminus of the HspA protein, yielding an extra sequence encoding about 2 kDa polypeptide added to HspA. Enterokinase (Boehringer Mannheim) digestion was performed to remove the His-tag. However, $HspA_{His}$ itself was degraded during enterokinase treatment (data not shown).

The oligomeric structure is characteristic for small heat shock proteins. The oligomer is the functional unit. The mechanism by which small heat shock proteins interact with unfolded proteins and thus prevent their aggregation is unknown. Leroux R. M. (Leroux *et al*., 1997b) established a model to explain the structure-function relationship of small heat shock proteins using Hsp16.2 from *C. elegans* as a model system (Fig. 3.2).

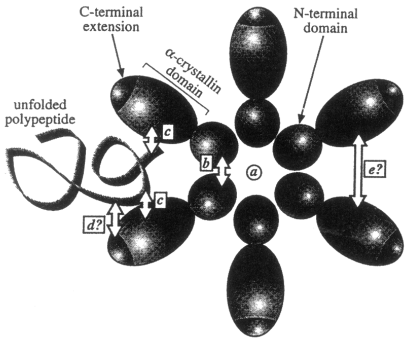


Fig. 3.2. Model for sHSP oligomeric structure and interaction with unfolded polypeptides (Leroux *et al*., 1997b). a, the proposed central cavity; b, N-terminal domain interactions; c, the interaction site between sHSP and unfolded protein; d, whether C-terminal extension is involved in the function of sHSP is unknown; e, how the subunits of sHSP assemble cooperatively into an oligomer is unknown.

According to this model, the N-terminal domain of sHSPs is buried in the central cavity as a consequence of the assembly of small heat shock proteins. The Hsp16.2 with poly His–tag was compared with the recombinant wild-type Hsp16.2. Both proteins assembled into

large oligomeric complexes although the size of the oligomers were somewhat different. The molecular mass of the Hsp16.2 wild-type oligomer is 550 kDa, while the size of $Hsp16.2_{His}$ is 680 kDa, as judged by SEC. In addition, the $Hsp16.2_{His}$ exhibited the same chaperone activities as the wild-type Hsp16.2 *in vitro*, suggesting that the N-terminus of Hsp16.2 can accommodate at least an additional 4-kDa of heterologous sequence per subunit without affecting its chaperone properties. Native His-tagged Hsp16.2 could not bind to Ni-agarose affinity resin.

In the case of $HspA_{His}$, the His-tagged HspA formed also a large oligomeric complex, which can interact with unfolded CS. However, in contrast to $Hsp16.2_{His}$, native $HspA_{His}$ binds to Ni-agarose affinity resin. This means that the assembly of $HspA_{His}$ does not match this model. How the N-terminal domain is arranged in the oligomeric complex of small heat shock proteins is far from clear. As mentioned in the Introduction, the N-terminal 32 residues are disordered in the complex of MjHSP16.5.

3.2.3. The chaperone activities of $HspA_{His}$

$HspA_{His}$interacts with chemically unfolded CS to prevent its aggregation in the solution. Bovine α-carystallin and mouse Hsp25 do not prevent chemically unfolded CS aggregation. In contrast to $HspA_{His}$, α-crystallin and Hsp25 suppress the aggregation of the unfolded insulin B-chain. In the course of insulin B-chain unfolding, partial structured intermediates are present. At the beginning of CS refolding process, the chemically denatured CS is completely unfolded. This suggests that at least some small heat shock proteins interact selectively with certain structures of the substrate. Hsp16.2 from *C. elegans* can interact with both thermally denatured and chemically denatured CS. It has reduced selectivity for the substrate structure.

3.2.4. The interaction of $HspA_{His}$ with unfolded CS

$HspA_{His}$prevents the reactivation of unfolded CS, suggesting that a stable complex is formed between $HspA_{His}$and unfolded CS. Such complexes may be productive or dead-end intermediates.

The chaperone function of small heat shock proteins may be cooperative with other chaperones as shown in the case of murine Hsp25 (Ehrnsperger *et al*., 1997) and *E. coli* IbpB (Veinger *et al*., 1998). When the complex formed by Hsp25 and thermally denatured CS was supplemented with Hsp70 and ATP, about 15% of CS were reactivated. Heat denatured malate dehydrogenase (MDH) was released from IbpB-MDH complex after supplementation

of DnaK/DnaJ/GrpE (KJE) chaperones and ATP. About 10% reactivation of MDH was observed. Moreover, GroEL/GroES (LS) chaperonines accelerated the rate of KJE-mediated refolding of IbpB-released MDH. Refolding of urea-heat denatured lactate dehydrogenase displayed a similar dependence on the IbpB/KJE/LS chaperone network.

In the case of $HspA_{His}$ Hsp70 and ATP did not cause the release of bound CS from the complex of $HspA_{His}$with unfolded CS. The cofactor for $HspA_{His}$ is unknown.

3.2.5. The physiological function of HspA

To elucidate the physiological function of HspA, a *hspA* deletion mutant was constructed. Four *hspA* deletion mutant strains were obtained. This small amount is due to the lower recombination efficiency when using linear DNA for transformation by electroporation. Three of the mutants showed a wild-type *S. aurantiaca* phenotype. One of the mutants showed abnormal rippling and an altered fruiting body (data not shown). M. Heidelbach described similar observations in his Inaugural-dissertation (ZMBH, Germany, 1992). Three strains with inactivated *hspA* were obtained in his work; one showed wild-type phenotype, the other two were unable to form fruiting bodies. Obviously, the alternation of the development of the *hspA* mutants is not due to the inactivation or deletion of *hspA* but due to unknown mutations since various phenotypes were detected. Furthermore, anti-HspA-sera did not affect the fruiting body formation of DW4/3-1 wild-type cells (data not shown).

As pointed out in the Introduction, small heat shock proteins from different organism have evolved different biological activities (Arrigo and Landry, 1994). The major small heat shock protein of *Saccharomyces cerevisiae* is Hsp26. Its expression is induced by heat shock, starvation or high concentration of salt. Another small heat shock protein identified in yeast cells is Hsp42 that shares high homology with Hsp26. In contrast to Hsp26, Hsp42 expression is more dependent on an increased salt concentration and on starvation. It is expressed also in unstressed cells. However, neither disruption of the gene encoding HSP26 nor a *hsp26/hsp42* double mutant had a detectable phenotype, even under stress conditions (Petko and Lindquist, 1986; Wotton *et al*., 1996).

The physiological role of HspA in *S. aurantiaca* is still enigmatic and the *hspA* deletion mutant did not offer any clue. However, the *in vitro* chaperone assay proved that $HspA_{His}$ has chaperone properties, suggesting HspA may have also a protective function *in vivo*.

Immunoelectron microscopy revealed HspA to be mainly distributed along the cytoplasmic membrane or the cell wall of the stressed cells. But, it was impossible to localise

HspA differentially in the outer membrane or at the inner side or outside of the cytoplasmic membrane by immunoelectron microscopy.

Periplasm is an important compartment of gram-negative bacteria that participates in cell physiological functions, e.g. solute transport and protein secretion. It has been observed that some periplasmic substrate-binding proteins have chaperone like properties. In addition to their role in transport and chemotaxis, they might help protein folding and renaturation in the periplasm (Richarme and Caldas, 1997).

During vegetative growth and development, myxobacteria secrete numerous extracellular proteins including proteinases and developmental signals (Dworkin, 1996). For example, the C signal has been demonstrated to be localised in the extracellular matrix by immunogold electron microscopy. Possibly, it is associated with the extracellular fibrils. The C signal as well as the spore coat protein U have a signal peptide for secretion (Gollop *et al*., 1991; Shimkets and Rafiee, 1990), while another spore coat protein S has no signal peptide. Myxobacteria have two extracellular appendages: pili and fibrils. Other proteins involved in gliding and cell-cell interaction should be expressed on the cell surface. It is possible that HspA plays a role in the stabilisation of extracellular or periplasmatic proteins under heat shock or in protein folding in the course of development.

3.3. The *ephA* gene, another indicator of eukaryote-like property?

The aspect of eukaryote-like properties of myxobacteria was reviewed by M. Dworkin (Dworkin, 1996). This view is based on the presence of serine-threonine kinases in *M. xanthus*, the sequence similarity between the spore protein S and ß/γ crystallin of vertebrate eye lens, and between the C signal and human 17 ß-hydroxysteroid dehydrogenase, the possible existence of a G protein in *S. aurantiaca*, and the presence of reverse transcriptase in myxobacteria, etc.

In this work, the ORFs of *hspA* and *ephA* were defined. The HspA shows homology to the plant small heat shock proteins. Interestingly, the sequence of EphA shows about 33% sequence identity to the vertebrate microsomal epoxide hydrolase, while only 13.9% to the epoxide hydrolase of *Agrobacterium radiobacter* and 12.2% to the epoxide hydrolase of *Mycobacterium tuberculosis*. In addition, the putative highly conserved catalytic residues in EphA of eukaryotes are conserved in the EphA of *S. aurantiaca*. All these data suggest the EphA of *S. aurantiaca* to be phylogenetically more closely related to the epoxide hydrolase of eukayotes than to those of prokaryotes.

3.4. Perspectives

The understanding of stress response in myxobacteria is just at the beginning. To further elucidate the transcriptional regulation of *hspA* gene and understand the function of HspA protein, the following investigations should be carried out:

- Identification of the sigma factor(s) that are involved in *hspA* transcription. As the sequence of different sigma factors share some conserved regions, part of the gene encoding the heat shock sigma factor can be detected using degenerated oligonucleotides that deduced from the conserved sequences. Using these primers in RT-PCR with RNA from heat shocked cells as template, it should be possible to detect the heat shock induced sigma factor genes.
- Purification of the putative enhancer protein(s). Study of the enhancer(s) might further elucidate the general mechanism for heat shock response in *S. aurantiaca*. The activator protein(s) may be obtained by affinity purification on streptavidin coated magnetic beads, to which the G3 fragment is bound *via* a distal biotin label.
- Isolation of other heat shock proteins from *S. aurantiaca*. As the transcriptional regulation of different heat shock genes in the same bacterium share some common pattern, study of other heat shock genes may improve the understanding of the regulation of *hspA* and the general heat shock response in *S. aurantiaca*. The common chaperone machinery in bacteria, such as DnaK, DnaJ, GrpE, GroEL, and GroES, has been studied in some other bacteria. Such chaperones can be cloned from *S. aurantiaca* also by the approach of degenerated PCR.
- Determination of the possible difference between $HspA_{His}$ and the wild-type HspA on both structure and function aspects. Although the His-tagged Hsp16.2 from *C. elegans* shows the same chaperone properties as wild-type Hsp16.2 (Leroux *et al*., 1997b), it is not yet clear whether it is true in the case of HspA from *S*. *aurantiaca*. The *hspA* should be expressed in *E. coli* using an expression vector without His-tag. The oligomeric structure and chaperone properties of $HspA_{His}$ and wild-type HspA should be analysed in parallel.
- Determination of the possible cooperator(s) of HspA. It should be tested whether other chaperones from S. *aurantiaca* can facilitate the function of HspA when they are known.
- Determination of the possible substrate(s) of HspA. The native substrate of *hspA* should be purified. As *hspA* was co-purified with the membrane fraction of *S. aurantiaca* cells (personal communication with H. U. Schairer, ZMBH, Germany), this substrate may be

found in the membrane fraction when using a $HspA_{His}$ coated Ni-agarose affinity column to separate this fraction.

IV. Materials and Methods

4.1. Materials

4.1.1. Chemicals

1-Butanol	Merck
3-(N-Morpholino)-propanesulfonic acid (MOPS)	Serva
5-Bromo-4-chloro-3-Indolyl-β-D-galactopyranoside (X-gal)	Biomol
5-Bromo-4-chloro-3-Indolyl phosphate (BCIP)	Sigma
Acetyl phosphate	Sigma
Acrylamide (2 x crist.)	Serva
Agarose	Sigma
Amberlite MB2	Serva
Ammonium peroxodisulfat (APS)	Serva
Bacto agar	Difco
Bacto yeast extract	Difco
Bacto tryptone	Difco
Bovine serum albumin (BSA)	Sigma
Bromphenol-blue	Serva
Diethyl pyrocarbonate (DEPC)	Sigma
Dithiothreitol (DTT)	Serva
Coomassie brilliant blue G250	Serva
Ethanol	Merck
Ethidium bromide	Serva
Etylenedinitrilo tetraacetic acid (EDTA)	Serva
Ethylene-bisoxyethyleneitrilo tetraacetic acid	Sigma
Ficoll 400	Pharmacia
Formaldehyde	Merck
Formamide	Merck
Glycerol	Merck
Glycine	Merck
Guanidine hydrochloride (GuHCl)	Gerbu
Indole	Sigma
Isoamyl alcohol	Merck
Isopropanol	Merck
Isopropyl thiogalactoside (IPTG)	Sigma
Low melting point agarose	Sigma
Magnesium sulfate	Merck
4-Methylumbelliferyl ß-D-galactoside (4-MUG)	Sigma
Nitro blue tetrazolium (NBT)	Sigma
N-2-Hydroxyethylpiperazin-N'-2 ethanesufonic acid (HEPES)	Roth
Ni^{+}-NTA-agarose	Qiagen

N’, N-Dimethylformamid	Sigma
N, N’-Methylene bisacrylamide	Pharmacia
N, N, N’, N’-Tetramethyl ethylerendiamine (TEMED)	Serva
Nonidet P40 (NP40)	Sigma
PEG 6000	Sigma
Phenol	Roth
Phenylmethlsulfonyl fluoride (PMSF)	Sigma
Polyvinyl pyrolidone	Sigma
Ponceau S	Sigma
Potassium acetate	Merck
Potassium chloride	Merck
Sackosyl	Merck
Sephadex G50	Pharmacia
Sodium acetate	Merck
Sodium chloride	Merck
Sodiumdodecyisulfate (SDS)	Serva
Tris base	Roth
Trytophan	Serva
Triton X-100	Serva
Urea	Roth
Xylene cyanol FF	Serva

4.1.2. Laboratorial equipment

Analytic balance	Sartorius
ß-ray counter Tri-carb 1500	Packard
Computer hardware	Power Macintosh 7600/132
Computer software	
Canvas 3.5	Deneba Software Inc
Cricket Graph III	Cricket Software
DNA Star	DNASTAR Inc
DNA strider 1.2	Commisariat a Énergie Atomique
EndNote 3,0	Niles & Associates, Inc
Entrez 2.0	National Centre for Biotechnology Informatics
MacPlasmap 2.05	CGC Scientific Inc
MacSPIRS 2.41	Silver Platter
MS Word 98	Microsoft Corporation
Netscape Communicator	Netscape Communication Cooperation
Oligo 4.0	National Biosciences Inc
Photoshop 3.05	MacApp Inc
Centrifuge	MSE

Chromatography system	LKB
Fastblot	Biometra
Fluorescence cells	Hellma
Fluorescence spectrophotometer MPF 44A	Perkin Elmer
French press	Amicon
Electroelution apparatus	Schleicher & Schuell
Electrophoresis apparatus	ZMBH
Electrophoresis constant power supply	Pharmacia
Gel dryer (DrygelSr.)	Hoefer Scientific Instruments
GenePulser (Pulse Controller)	Bio-Rad
Gilson pipetman	Gilson
Heat block	Liebish
HPLC system	Pharmacia
Microcamera MPS 60	Leica
Microcells	Ratiolab
Micromicroscpoy M 420	Leica
PhosphoImager Fuji BAS 1000	Fuji Inc
Quartz cells	Hellma
Sequence analysis system	LKB
Speed-vac-concentrator Savant	Bachofer
Spectrofluophotometer RF 5000	Shimadza
Spectrophotometer	Kontron
Sonifier B15	Branson
Table centrifuge Biofuge A	Heraeus
Themocycler (ThioThermobloc)	Biometra
Thermostate	Julabo
Trio-thermoblock	Biometra
Ultracentrifuge L8-70M	Beckman
Ultrafiltration system	Millipore
UV-stratalinker	Stratagene
X-ray film developer	Amersham
Vacuum blotter	Appligene

4.1.3. Consumables

Biodyne B blotting membrane	Pall
Centriprep 10	Amicon
Nitrocellulose membrane	Appligene
Nylon filter	Millipore
X-ray film	Kodak

4.1.4. Radioisotope

γ-^{32}P-ATP (5000 Ci/mmol, 10 mCi/ml)	Amersham
α-^{32}P-dATP (3000 Ci/mmol, 10 mCi/ml)	Amersham
α-^{35}S-dATP (1000 Ci/mmol, 10 mCi/ml)	Amersham

4.1.5. Enzymes and kits

Alkaline phosphatase (CIP)	Boehringer
AMV reverse transcriptase	Boehringer
Citrate synthase (CS)	Boehringer
DNA polymerase Klenow I fragment	Boehringer
Insulin	Sigma
Lysozyme	Sigma
Restriction endonuclease	Boehringer
	Biolabs
	Promega
RNase A	Serva
RNase inhibitor	Promega
T4 polynucleotide kinase	Biolabs
T4 DNA ligase	Boehringer
Vent DNA polymerase	Biolabs
GeneClean II Kit	Bio 101
Nick Translation Kit	Boehringer
Plasmid purification Kits	Qiagen
	Nucleobond
Protein assay kit	BioRAD
Sequenase version 2.0 DNA sequence kit	Amersham

4.1.6. Nucleic acids

4.1.6.1. Oligonucleotides

2'-Desoxyribonucleotide (dNTPs)	Amersham
2'-Desoxyribonucleotide 5'-O-(1-thiotriphosphates) (α-S dNTPs)	Amersham

4.1.6.2. PCR-Primers (5'-3')

A11:	GCGCGTCGACGATGACGATGACAAAATGGCCGATTTGTCTGTTC
A12:	GGCAAGCTTCATGAAAAGCCTGGCCAC
B1:	GCATCTGCTGAAAGGGATCC
B2:	CGGGATCCTCGGGGCCGTCCCTGCCTA
B3:	CGGGATCCGCTTCACGCTCGCGTCCCTT
B4:	CGGGATCCGACGGTTCCTCCTCTCGTAT

C2: CCATCGATCGGCGCACCGGCTACGAGAC

C3: CCATCGATCGGTGGACCAGGCGACGGTGA

C6: CCATCGATCCTGGTCCACCGCCTGCTTC

C9: CCATCGATGGGACGCGAGCGTGAAGCGC

C11: CCATCGATCTTGCGCTGTCTCGAAGCTG

C12: CCATCGATACGGCATGGCCTCGGGGCCG

C13: CCATCGATTGGATGGCGGCGGCAGTCCA

C14: CCATCGATTACGAGACCACGTCCAAGGAGA

E3: CGGAATTCCTGGAGGCGAATAGAATTTCA

E5: CGGAATTCACCAGGCGACGGTGAGCAACGGT

KO-*Bam*HI: CGGGATCCAAGGAACACATCAAGGCGTAGC

KO-*Cla*I: CCATCGATATGACGGTTCCTCCTCTCGTAT

KO-*Eco*RI: GGAATTCACTTCGTCTCGACTTCGGGGAA

KO-*Sal*I: ACGCGTCGACAACCGTTTCCCGAACTACCGCA

S4E: CGGAATTCTAGCCGGTGCGCCGCCTGGGAA

S5E: GGAATTCGAGGCCATGCCGTCTTGCGCT

G1:

E1: CGGAATTCACAGCCAGACGCAGGGCAGACAT

Gel 1-3: TCGAGACAGCGCAAGACGGCAT

G2:

Gel 2-5: GCTGGAGGCGAATAGAATTTC

SHB 4: See 4.1.6.3

G3:

Gel 4-5: GCCGGTGCGCCGCCTGGGAAGCAG

Gel 5-3: CGTCCCTTGGGCCGTGTGTTAA

4.1.6.3. Sequence-Primers (5’-3’)

SHA 1: AAGAATGGGGTGTTGACGCT

SHA 2: AAGTAACGTGCGCTCAT

SHA 3: GGTCGGGTTCGTTCCAGTAG

SHA 4: AAGCGCTGGGCCGAGCAGAG

SHA 5: TGTTGTCGAGCATCTCGTCC

SHA 6: GATTTACGAGAAGTTCCAAG

SHA 7: AAGCTTCTCCGGGAAGACGA

SHA 8: CTGAACTTTCCGCTCGTCTT

SHA 9: AGAATCGGCAGGGCGTTCTCAT

SHA 10: GTCCAAGCATGAGAACG

SHA 11: CGTTCAAGATTGCCGTCC

SHA 12: ACGGCAATCTTGAACGG

SHA 13:	CATGGTTTTAGTGGACG
SHA 14:	CGTCCACTAAAACCATG
SHB 1:	TACATCTTCAAGGCGGACCT
SHB 2:	TCCTGCATCTGCTGAAAGGG
SHB 3:	TGTCTCGAAGCTGTCTTACG
SHB 4:	AGACCACGTCCAAGGAGACG
SHB 5:	AGCAACGAGCTGGTGCTCTA
SHB 6:	TTGGGCAACGCGACGATGCC
SHB 7:	CAATGCGTTCCTCAAAGAGG
SHB 8:	AGTTCGTCCTTGCGTGCCTT
SHB 9:	AGGAGAACGCGAACCTGA
SHB 10:	TCAGGTTCGCGTTCTCCT

4.1.6.4. Linkers (5'-3')

MCS in #9:

#9-MCS-1:	CGGAATTCGGGATCCCAAGCTTGCTCTAGACCGC
#9-MCS-2:	TCGAGCGGTCTAGAGCAAGCTTGGGATCCCGAATTCCGGTAC

MCS in modified pSUP102:

HUS 2001:	AGCTTCGGGATCCACGCGTGTCGACGGTACCACTAGTGCGGCCGCATGGGCC
HUS 2002:	GGCCCATGCGGCCGCACTAGTGGTACCGTCGACACGCGTGGATCCCGA

4.1.6.5. Vectors and plasmids

pBC SK+	Stratagene
pBluescript SK-	Stratagene
pBR 322	Biolabs
pQE9	Qiagen
pSUP102	(Simon *et al*., 1986)
mini Tn5 lacZ1	(de Lorenzo *et al*., 1990)
pUC4 KIXX	Pharmacia

4.1.6.6. DNA markers

DNA marker II	Boehringer
DNA marker III	Boehringer
DNA marker VI	Boehringer

4.1.6.7. RNA marker

RNA marker I	Boehringer

4.1.6.8. Others

poly [d (I-T)]	Boehringer
Salmon sperm DNA	Sigma
tRNA from baker's yeast	Boehringer

4.1.7. Proteins

4.1.7.1. Antibodies

Anti rabbit IgG, alkaline phosphatase conjugated Sigma

4.1.7.2. Protein Marker

MW-SDS-70L kit Sigma

4.1.8. Bacterial strains

4.1.8.1. *E. coli* strains

XL-1 Blue MRF'	Δ *(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac* (F', *proAB, lacI*^{q}Z ΔM15, Tn10, (tet^{r})). Stratagene.
JM110	*rpsL* (str^{r}), *thr, leu, endA, thi-1, lacY, galK, galT, ara, tonA, tsx, dam, dcm, supE44,* Δ (*lac-proAB*) (F'*traD36, proAB, lacI*^{q}*Z* ΔM15). Stratagene.
M15 (pREP4)	Nal^{s}, Str^{s}, Rif^{s}, *lac*$^{-}$, *ara*$^{-}$, *gal*$^{-}$, *mtl*$^{-}$, *F*$^{-}$, *recA*$^{+}$, *uvr*$^{+}$. Qiagen.
GI 698	F^{-}, λ^{-}, *lac*I^{q}, *lac*PL8, *amp*C::P$_{trp}$cI. Invitrogen.

4.1.8.2. *S. aurantiaca* strain

DW4/3-1	Wild type, Str^{r} (Qualls *et al*., 1978)

4.1.9. Media and stock solutions

4.1.9.1. Media

LB medium:

Bacto tryptone	1%
Bacto yeast extract	0.5%
NaCl	1%
pH 7.2	

Tryptone medium:

Bacto tryptone	1%
$MgSO_4$ $7H_2O$	0.2%
pH 7.2	

Water agar medium:

$CaCl_2$	0.1%
Bacto agar	1.5%

4.1.9.2. Stock solutions

Antibiotics: 1000 ×

Ampicillin sodium salt	100 mg/ml in H_2O
Chloramphenicol	25 mg/ml in 100% Ethanol
Kanamycin sulphate	50 mg/ml in H_2O
Tetracycline hydrochloride	10 mg/ml in 100% Ethanol
Streptomycin sulphate	125 mg/ml in H_2O

Other solutions:

Ethidium bromide solution (20,000 ×):	10 mg/ml in H_2O
IPTG stock solution	100 mM in H_2O
X-gal stock solution	20 mg/ml in dimethylformamid

20 × SSC:

NaCl	3 M
Sodium citrate	0.3M
PH 7.0	

50 × Denhardt's solution:

Ficoll	1%
Polyvinyl pyrolidone	1%
BSA	1%

TE buffer:

Tris-HCl, pH 8.0	10 mM
EDTA	1 mM

Enzyme buffer:

10 × A, B, H, L, M buffer	Boehringer
10 × NEB 1, 2, 3, 4 buffer	Biolabs
5 × AMV buffer	Promega
10 × Ligase buffer	Boehringer
10 × PNK buffer	Boehringer
10 × CIP buffer	Boehringer
10 × RNase buffer	Boehringer

50 × TAE buffer:

Tris-acetate	2 M
EDTA	0.05 M
pH 8.0	

10 × TBE buffer:

Tris-borate	0.89 M
EDTA	0.02 M
pH 8.0	

4.2. Methods

4.2.1. Microbiologic techniques

4.2.1.1. Growth of *E. coli*

E. coli was grown in liquid LB medium or on LB agar (1.5% agar) plates at 37°C. Antibiotic concentrations for *E. coli* were as follows: ampicillin sodium salt 100 μg/ml, kanamycin suphate 50 μg/ml, tetracycline hydrochloride 10 μg/ml, and chloramphenicol 25 μg/ml.

4.2.1.2. Growth of *S. aurantiaca*

S. aurantiaca was grown in liquid Tryptone medium or on Tryptone agar (1.5% agar) plates at 32°C. Antibiotic concentrations for *S. aurantiaca* were as follows: streptomycin sulphate 125 μg/ml, kanamycin sulphate 50 μg/ml.

4.2.1.3. Heat shock induction of *S. aurantiaca*

S. aurantiaca was grown in liquid Tryptone medium at 28°C until a log phase was reached (1.6×10^8 cells/ml). Then the culture was shifted to a 38°C water bath with vigorous shaking.

4.2.1.4. Spore induction in *S. aurantiaca* by indole

S. aurantiaca was grown in liquid Tryptone medium at 28°C until a late log phase was reached (2.2×10^8 cells/ml). Then indole (1 M in 100% ethanol) was added to the culture to a final concentration of 0.5 mM.

4.2.1.5. Fruiting body formation of *S. aurantiaca*

S. aurantiaca was grown in liquid Tryptone medium at 32°C until a log phase culture was reached (2×10^8cells/ml). Cells were harvested by centrifugation at 4,000 × g for 15 min at 4°C. The cell pellet was washed twice with 10 mM HEPES, 0.5 mM $CaCl_2$ (pH 7.2) buffer and then resuspended in the same buffer to a final concentration of 4×10^{10} cells/ml. 5-10 μl of the cell suspension was spotted on water agar plates. After drying, the plate was incubated at 32°C for 24 hours or longer. To analyse the temperature dependency of fruiting body formation, the assay was performed at different temperatures (30°C, 32°C, 35°C, 37°C, and 39°C) synchronously and the fruiting body formation was controlled after different time periods.

4.2.1.6. Preservation of *E. coli* and *S. aurantiaca* cultures

E. coli was grown to a log phase and 0.8 ml of the culture was transferred to a sterile tube and mixed with 0.2 ml of sterile glycerol. The culture was preserved at -80°C.

S. aurantiaca was grown to a log phase and 1 ml of the culture was transferred into a sterile tube and frozen with liquid nitrogen; or 1.5 ml of the culture was transferred to a sterile tube and spun down at 4,000 × g for 5 minutes. The cell pellet was resuspended in 0.4 ml liquid Tryptone medium containing 25% glycerol. The culture was preserved at -80°C.

4.2.1.7. Germination of *S. aurantiaca* spores

S. aurantiaca fruiting body formation was performed on water agar plates with filter papers. After the fruiting bodies matured on the filter papers, the fruiting-body containing filter papers were dried by incubating

with Silicon gel for one month at room temperature in a closed desiccator. The filter papers were then transferred on Tryptone agar plates (upside down) and incubated at 32°C for two days.

4.2.1.8. Electroporation of *E. coli*

Electrocompetent cells were prepared with 1 litre of *E. coli* culture in LB medium, which was inoculated with 1/100 volume of fresh overnight culture and incubated at 37°C with vigorous shaking until the OD_{600} value reached 0.5 to 1.0. After chilling down on ice for 30 min, cells were harvested by centrifugation at 4,000 × g for 15 min at 4°C. The cell pellet was washed once with 1 litre of cold H_2O, once with 0.5 litre of cold H_2O and once with 20 ml of cold 10% glycerol. After resuspending the cells in cold 10% glycerola to a final volume of 2 to 3 ml, they were dispensed into 40 µl portions, frozen in liquid nitrogen and stored at -80°C.

For electroporation, 10 to 50 ng DNA dissolved in 1 to 5 µl of a low ionic strength buffer was mixed with the thawed electrocompetent cells in an eppendorf tube and the tube was kept on ice for about 1 min. The cell-DNA mixture was then transferred to a cold electroporation cuvette (Gap distance is 1 mm). Electroporation was performed with the BioRad GenePulser with a field strength of 12.5 kV/cm, an electric capacity of 25 µF and a resistance of 200 Ω. After electroporation, 1 ml of LB or SOC medium was added into the electroporation cuvette immediately. The cell suspension was transferred to a test tube and incubated at 37°C with vigorous shaking for one hour. Then the cells were spreaded onto LB agar plates containing appropriate antibiotics.

SOC medium:

Tryptone	2%
Yeast extract	0.5%
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM

4.2.1.9. Electroporation of *S. aurantiaca* (Stamm *et al.*, 1999)

The electrocompetent cells were prepared freshly with a log phase culture of *S. aurantiaca*. The cells were harvested by centrifugation at 4,000 × g for 15 min at 20°C. The cell pellet was washed once with an equal volume of 5 mM HEPES, 0.5 mM $CaCl_2$ buffer, pH 7.2, at room temperature, following with 1/2 volume of the same buffer. The cells were then resuspended in the same buffer to a final concentration of about 4×10^{10} cells/ml. About 0.1 to 0.5 µg DNA was mixed with 40 µl competent cells. Electroporation was performed with the BioRad GenePulser with a field strength of 8.5 kV/cm, an electric capacity of 25 µF and a resistance of 200 Ω. Thereafter, the *S. aurantiaca* cells were grown in 50 ml of liquid Tryptone medium without selective antibiotic at 32°C for 20 hrs. The cells were harvested by centrifugation at 4,000 × g for 15 min at 4°C and resuspended in 2 ml of 0.1 M HEPES, pH 7.2, 10 mM $CaCl_2$ buffer. 0.1-0.5 ml of the cell solution was mixed with 3 ml of pre–warmed (42°C) soft agar Tryptone medium (0.75% agar, containing appropriate antibiotics) and then placed on a Tryptone agar plate containing appropriate antibiotics. The plates were incubated at 32°C for 5 to 7 days.

4.2.1.10. White-blue colony selection of *E. coli*

Some cloning vectors (e.g. pBC SK+ and pBluescript SK-) carry the regulatory sequences and the first 146 amino acids of the coding region of the ß-galactosidase gene. A polycloning site is embedded in the coding region. After transferring into *E. coli* host cells that code for the carboxy-terminal portion of ß-galactosidase, the

active ß-galactosidase will be obtained due to the complementation of the N- and C- portions of ß-galactosidase. Blue colonies will appear in the presence of chromogenic substrate X-gal. When foreign DNA is inserted into the multiple cloning site of the vector, the N–terminal portion will be disrupted and white colonies appear.

To select such recombinant clones, bacteria cells were spreaded on LB agar plates that were placed by 40 µl of X-gal stock buffer and 40 µl of IPTG stock buffer.

4.2.1.11. Expression of fusion protein in *E. coli* M15 and GI698

E. coli M15 cells carrying pQE-expression plasmid were grown in LB medium at 37°C overnight. A fresh culture was inoculated with the overnight culture at a ratio of 1:50 and then incubated at 37°C with vigorous shaking until a log phase (OD_{600} = 0.7-0.9) was reached. IPTG was added to the culture to a final concentration of 1-2 mM. The maximum expression of fusion protein was obtained after about 3-5 hrs.

When using *E. coli* GI698 to express the fusion protein, the cells carrying pQE-expression plasmid were grown in 10 ml of RM medium at room temperature overnight. After inoculation with the overnight culture at a ratio of 1:20, the fresh culture in Induction medium was grown at room temperature with vigorous shaking until a log phase (OD_{550} = ca. 0.5) was reached. IPTG and tryptophan were added to the culture to a final concentration of 2 mM and 100 µg/ml, respectively. The maximum expression of fusion protein was obtained after about 5 hrs.

RM medium (Invitrogen):	
Na_2HPO_4	0.6%
KH_2PO_4	0.3%
NaCl	0.05%
NH_4Cl	0.1%
Casamino acids	2%
$MgCl_2$	0.0095%
PH 7.0	
Induction medium (Invitrogen):	
Na_2HPO_4	0.6%
KH_2PO_4	0.3%
NaCl	0.05%
NH_4Cl	0.1%
Casamino acids	0.2%
$MgCl_2$	0.0095%
PH 7.0	
Tryptophan solution:	
Tryptophan	10 mg/ml in H_2O

4.2.2. Isolation and manipulation of DNA

4.2.2.1. Preparation of plasmid DNA from *E. coli* cells

I. Mini-prep

A single colony of *E. coli* was transferred into 2 ml of LB medium containing appropriate antibiotic. The culture was incubated at 37°C overnight. Cells were spun down with a table centrifuge. The cell pellet was resuspended in 300 µl of STET buffer in an eppendorf tube. 20 µl of 10 mg/ml lysozyme solution was added. The tube was incubated at room temperature for 20 min and then transferred in a beaker of boiling water. It was held in the boiling water for two min. After centrifugation at 10,000 × g for 10 minutes at 4°C, the plasmid DNA containing supernatant was transferred to a new tube and precipitated with 500 µl of 75% isopropanol/2.5 M NH_4Ac solution at room temperature for 20 min. The sediment of plasmid DNA was obtained by centrifugation at 10,000 × g for 15 min at 4°C. DNA was washed once with 75% ethanol and dried under vacuum. The plasmid DNA was dissolved in 50 µl of TE buffer.

STET buffer:

Sucrose	250 mM
Tris-HCl, pH 7.5	50 mM
EDTA, pH 8.0	50 mM
Triton X-100	5%

II. Preparation of plasmid DNA with a kit

Plasmid DNA was isolated *via* Plasmid purification Kits supplied by Qiagen and Nucleobond according to the recommended protocols of the manufacturers.

4.2.2.2. Isolation of genomic DNA from *S. aurantiaca*

The method described by Meade (Meade *et al*., 1982) was modified for the isolation of total DNA from *S. aurantiaca*. Cells from 200 ml of log phase culture were harvested by centrifugation at 4,000 × g for 15 min at 4°C. Cell pellet was resuspended in 50 ml of 1 M NaCl buffer, and the suspension was agitated gently at 4°C for one hour. After centrifugation, the cell pellet was washed once with 25 ml of cold TES buffer (10 mM Tris-HCl, 2.5 mM EDTA, 150 mM NaCl, pH 8.0) and then resuspended in 15 ml of TE buffer (10 mM Tris-HCl, 2.5 mM EDTA, pH 8.0). 1.5 ml of lysozyme solution (2 mg/ml lysozyme in the TE buffer) was added. After incubation at 37°C for 15 min, 2 ml of Sarkosyl-pronase solution (2 mg/ml proteinase K in 10% Sarkosyl solution) was added. The mixture was incubated for an additional hour at 37°C. Lysed cells were extracted once with Tris–saturated phenol (pH 8.0), once with phenol/chloroform/isoamyl ethanol (25:24:1), and once with chloroform/isoamyl ethanol (24:1). The DNA in aqueous phase was precipitated with an equal volume of isopropanol containing 0.3 M sodium acetate (pH 5.2) at room temperature and washed once with 75% ethanol. After drying under vacuum, DNA was dissolved in 300 µl of TE buffer at 55°C overnight.

4.2.2.3. Phenol extraction

The standard procedure was used to remove protein from a DNA solution (Sambrook *et al*., 1989). An equal volume of Tris-saturated phenol was added to the DNA solution and mixed with it. After centrifugation at 10,000 × g for 5 min at room temperature, the upper aqueous phase was carefully transferred to a clean tube and

extracted once with an equal volume of phenol/chloroform/ isoamylalcohol (25:24:1) and once with an equal volume of chloroform/isoamylalcohol (24:1). The final DNA solution was then precipitated with ethanol or isopropanol.

4.2.2.4. Precipitation of DNA with ethanol or isopropanol

3 M sodium acetate solution (pH 5.2) was added to a DNA solution to a final concentration of 0.3 M. The DNA sample was precipitated with 2 volumes of cold ethanol (99%) at -20°C for 2 hours or with 0.7 volume of isopropanol at room temperature for 15 min. DNA was recovered by centrifugation at 10,000 × g for 15 min at 4°C and washed with 75% ethanol. The DNA pellet was dried under vacuum and dissolved in TE buffer.

4.2.2.5. Determination of the purity and the concentration of a DNA solution.

The purity and the concentration of a DNA preparation were determined by the spectrophotometric measurement. The ratio between the absorbance at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the preparation: a pure DNA solution has an OD_{260}/OD_{280} ratio of 1.8. The concentration of the DNA solution was calculated by the OD_{260} value:

1 OD_{260} = 50 μg /ml double-stranded DNA

1 OD_{260} = 33 μg /ml single-stranded DNA

4.2.2.6. DNA restriction and ligation

DNA restrictions were performed according to the standard protocols (Sambrook *et al.*, 1989). The reaction conditions were recommended by the manufacturers.

Ligation of double-stranded DNA was carried out using T_4-DNA ligase. The reaction was as follows:

0.1 μg vector DNA

0.3 μg insertion DNA

1 μl of 10 × ligase buffer

10 Weiss-units T_4 ligase

H_2O was added to a final volume of 10 μl

The reaction was incubated at 15°C overnight or at room temperature for 2 hours.

4.2.2.7. DNA electrophoresis

Horizontal agarose gel electrophoresis was usually performed to separate and analyse different sizes of DNA fragments (Sambrook *et al.*, 1989). A 0.6-2.0% agarose gel in 1 × TAE buffer was used depending on the sizes of the fragments to be separated. Electrophoresis was performed at a voltage of 1-5 V/cm. After electrophoresis, the gel was stained with 0.5 μg/ml ethidium bromide for 5 min and the DNA bands were visualised on a transilluminator (254 nm) and recorded with a video copy processor of Mitsubishi.

2.2.8. Recovery of DNA fragments from agarose gel

I. Recovery of DNA fragments from agarose gel with GeneClean II kit

The GeneClean II kit was used to recover DNA whose size is in the range of 500 bp to 10 kbp. The agarose gel strip containing the target DNA fragment was cut out and transferred to an eppendorf tube. 3 volumes of NaI stock solution were added and the tube was incubated at 55°C for about 5 min to dissolve agarose. Glassmilk

suspension (5 µl/µg DNA) was added to the DNA solution. After incubation on ice for 5 min, the glassmilk/DNA complex was spun down by centrifugation at 10,000 × g for 1 min. The pellet was washed thrice with cold New Wash solution. The DNA absorbed by the glassmilk was eluted with water or low-salt buffer to a final concentration of about 10 µl/µg DNA.

II. Recovery of DNA fragments from agarose gel by electroelution

The electroelution method developed by Schleicher & Schuell was used to recover DNA fragments that are larger than 10 kbp. This method employed two kinds of membrane, BT1 and BT2. BT1 is an inert membrane. It allows only those molecules that are smaller than 6 kDa to go through. BT2 is a prefilter, which allows all charged molecules to go through but stops large particles, like agarose slices. In practice, BT1 and BT2 together form a trap, in which the biomolecules such as DNA or RNA to be recovered.

The apparatus was assembled according to the instruction supplied by the manufacturer. The electroelution was performed in 1 × TAE buffer at 200 V for 2 hours. The electric field was then reversed using the same voltage for further 20 seconds to remove the DNA molecules from the BT1 membrane. The DNA was precipitated with ethanol.

III. Recovery of DNA fragments from low melting temperature agarose gel

This method was used to recover DNA fragments that are smaller than 500 bp. DNA fragments were separated on a low melting agarose gel. The target gel strip was cut out and transferred to an eppendorf tube. 5 volumes of TE buffer were added to the tube and then the tube was incubated at 65°C for 5 min to dissolve the agarose. The DNA solution was then extracted once with an equal volume of phenol, once with an equal volume of phenol/chloroform, and once with an equal volume of chloroform. The DNA was precipitated with 2 volumes of cold ethanol containing 1 M NH_4Ac.

4.2.2.9. Dephosphorylation of DNA fragments

Dephosphorylation of DNA fragments was usually performed with a linearised vector DNA before ligation. For 5'-protruding DNA fragments, about 2.5 pmol DNA ends (1.25 pmol fragments) were incubated with 0.1 unit alkaline phosphatase at 37°C for 30 min in 1 × CIP buffer. For 3'–protruding or blunt-end DNA fragments, 1 unit alkaline phosphatase was used for the dephosphorylation of 1-2 pmol of DNA ends. The reaction was incubated at 50°C for 45 to 60 min and then stopped by addition of 2 µl of 0.5 M EGTA. After the reaction, alkaline phosphatase was denatured by heating up to 65°C for 20 min and DNA was purified by phenol extraction.

4.2.2.10. Fill-in of 5'-protruding ends of DNA fragments

For cloning a DNA fragment, if no appropriate restriction site is available within the vector DNA, the vector DNA and the DNA fragment (if both have 5'-protruding ends) can be filled-in to obtain blunt ends using the Klenow I fragment in the presence of dNTPs. The vector and the foreign DNA fragment can be ligated together using blunt-end-ligation. The 5'-protruding ends can also be partially filled-in so that appropriate sticky end can be generated between vector and foreign DNA fragments. The filling reaction was as follows:

0.1-1 µg DNA with 5'-protruding ends

2 µl of 10 × H buffer

1 µl of dNTP (2.5 mM/each)

2 units Klenow I fragment

Added H_2O to the final volume of 20 µl

The reaction mixture was incubated at 37°C for 30 min. The DNA was subsequently purified by phenol extraction (Sambrook *et al*., 1989).

4.2.2.11. Radioactive labelling of a DNA fragment

I. Labelling of DNA fragments using the Nick Translation kit

Labelling of double-stranded DNA fragment was performed by nick translation using the Nick Translation Kit and according to the recommended protocol. The standard reaction was as follows:

0.1-1 µg DNA (> 500 bp)

3 µl of dCTP, dGTP, dTTP mixture (0.4 mM for each)

H_2O was added to a final volume of 16 µl

2 µl of 10 × buffer

2 µl ^{32}P-dATP (3.000 Ci/mmol)

2 µl of enzyme mix (Klenow I fragment and DNase I)

The mixture was incubated at 15°C for 35 min, and the reaction was stopped by addition of 2 µl of 0.2 M EDTA (pH 8.0). The labelled DNA fragments were purified by gelfiltraton using a 1 ml Sephadex G-50 column.

II. Labelling of DNA probes by priming

Double-stranded DNA can be labelled by random priming or special priming. The standard reactions were as follows:

A. Random priming:

100 ng DNA

2 µl of 10 × Hexanucleotide-Mix (Boehringer)

The mixture was heated up to 100°C for 10 minutes and then chilled down on ice for 5 min

2 µl of 10 × H buffer

2 µl of dNTP mixture (2.5 mM for each)

H_2O was added to a final volume of 19 µl

2 units Klenow I

After incubating at 37°C for one hr, the reaction was stopped by heating up to 68°C for 20 min.

B. Special priming

100 ng DNA

100 pmol of special primers that are complemented to the sequence of the probe DNA

The mixture was heated up to 100°C for 10 minutes and then chilled down on ice for 5 min

2 µl dNTP (2.5 mM for each)

2 µl 10 × H buffer

H_2O was added to a final volume of 19 µl

2 units Klenow I

After incubating at 37°C for one hr, the reaction was stopped by heating up to 68°C for 20 min.

The labelled DNA was purified as described above.

4.2.2.12. DNA hybridisation techniques

I. Colony transfer and hybridisation for *E. coli*

Colony hybridisation is convenient to screen the target gene from a gene library. *E. coli* colonies were grown on LB plates until the diameter of a single colony was about 1 to 1.5 mm. A Biodyne B nylon membrane was placed on the plate for 20 seconds. The membrane was then transferred on 10% SDS-saturated Whatman paper for 3 min (the colony containing side was upwards), then on 0.5 M NaOH, 1.5 M NaCl buffer-saturated Whatman paper for 10 min, on 0.5 M Tris, pH 7.5, 1.5 M NaCl buffer-saturated Whatman paper for 10 min, and on 10 × SSC buffer-saturated Whatman paper for 10 min. After air drying and UV-cross linking, the membrane was washed with 50 mM Tris, pH8.0, 1 M NaCl, 1 mM EDTA, 0.1% SDS buffer for 20 min before hybridisation.

Colony hybridisation, Southern hybridisation and Dot hybridisation were carried out according to the standard protocols (Sambrook *et al*., 1989). Prehybridisation was carried out for one hour in the prehybridisation solution. Hybridisation was performed in hybridisation buffer overnight. The membrane was then washed twice with Wash Solution I at room temperature for 10 min and twice with Wash Solution II for 15 min at hybridisation temperature. The hybridisation temperature was 55°C to 60°C. The membrane was then autoradiographed.

Solutions for DNA hybridisation:

Prehybridisation solution:

5 × SSC

5 × Denhardt's solution

100 µg/ml denatured salmon sperm DNA

0.5% SDS

Hybridisation solution:

5 × SSC

5 × Denhardt's solution

100 µg/ml denatured salmon sperm DNA

0.5% SDS

^{32}P-labelled DNA probe

Wash solution I:

2 × SSC

0.1%SDS

Wash solution II:

0.2 × SSC

0.1% SDS

II. Dot blotting and hybridisation

Dot hybridisation was used to screen a target gene from a gene library or to screen target recombinants. 5 to 10 µg genomic DNA or 0.5 µg plasmid DNA were mixed with 2 µl of 1 M NaOH, 50 mM EDTA buffer and heated up to 100°C for 10 min. After chilling down on ice for 10 min, the DNA was spotted onto a Biodyne B

nylon membrane and air-dried for 15 min. After UV-cross linking, hybridisation was carried out as described above.

III. Southern transfer and hybridisation

Southern hybridisation was used to screen a restriction fragment of target DNA or to confirm the structure of recombinant DNA. DNA restriction fragments were separated on an agarose gel. After electrophoresis and photography, the gel was submerged in 0.25 M HCl solution with gentle shaking for 20 min at room temperature, then the DNA fragments were transferred onto a Biodyne B nylon membrane in 0.4 NaOH solution overnight. The transfer apparatus was set up according to the standard protocol (Sambrook *et al*., 1989). After UV-cross linking, hybridisation was carried out as described above.

4.2.2.13. Oligonulceotides purification

40 nmol of chemically synthesised oligonucleotides were resuspended in 100 µl of H_2O. 1 ml of 1-butanol was added. The solution was vortexed and centrifuged at 13,000 rpm for 5 min with a table centrifuge. The supernatant was discarded and the pellet was dissolved in 100 µl of water. The butanol extraction was repeated twice. After drying under vacuum, the pellet was dissolved in 300 µl of water and the concentration of the oligonucleotides was determined by spectrophotometry.

OD_{260} = total extinction coefficient (ε) (in a 1 cm path-length cuvette) × concentration (C)

ε for each of the bases is:

dGTP = 11.7 ml/µmole

dCTP = 7.3 ml/µmole

dATP = 15.4 ml/µmole

dTTP = 8.8 ml/µmole

4.2.2.14. Radioactive labelling of oligonucleotides

5'-End labelling of oligonucleotides was carried out using T_4 Polynucleotide kinase (PNK) and γ^{32}P-ATP according to the standard protocol (Sambrook *et al*., 1989). The reaction was as follows:

5 pmol oligonucleotides (>18 bp)

2 µl of 10 × PNK buffer

H_2O was added to the final volume of 17 µl.

The mixture was heated up to 70°C for 10 min and cooled down on ice for 5 min

2 µl γ^{32}P-ATP (3,000 Ci/mmol)

10 units of PNK

The reaction was incubated at 37°C for one hr and then stopped by addition of 2 µl of 500 mM EDTA. The solution was heated up to 65 °C for 10 min. The labelled oligonucleotides were purified by gel filtration using 1 ml of Sephadex G 50 column.

4.2.2.15. Amplification of DNA with polymerase chain reaction (PCR)

PCR was carried out using Vent polymerase. The standard reaction conditions were as follows:

Total volume	50 µl	100 µl
DNA template:	1-100 ng	1-100 ng
10 × polymerase buffer:	5 µl	10 µl

BSA (10 mg/ml)	0.5 µl	1 µl
Primer 1 (5'→ 3')	50 pmol	100 pmol
Primer 2 (3'→ 5')	50 pmol	100 pmol
10 × dNTP mix (2.5 mM for each)	5 µl	10 µl
Vent-polymerase	10 units	20 units
H_2O	to 50 µl	to 100 µl

The reaction mixture was overlaid with 50 µl of mineral oil. The conditions for the amplification with the Trio-Thermoblock were as follows: The initial denaturation was performed at 95°C for 5 min; the further 2 to 35 cycles were performed with recycling of denaturation at 95°C for 45 seconds, following with chain annealing at 55°C to 72°C for 45 seconds and chain extension at 72°C for 90 seconds. After incubating the reaction at 72°C for further 10 min to polish the PCR product, the PCR product was purified using a PCR purification kit (Qiagen). When more than one DNA fragments were obtained, the PCR product was separated by agarose gel electrophoresis and the target product was recovered from the agarose gel.

4.2.2.16. DNA sequencing

Double-stranded DNA was sequenced using Sequenase version 2.0 DNA sequencing kit (Amersham) by the Sanger method (Sanger *et al*., 1977). The samples were run on a 6% polyacrylamide gel containing 7 M urea for about 4 hrs with an electric power of 50 Watt. After electrophoresis, the gel was dried under vacuum and then autoradiographed. Analysis of sequence data was performed using the program DNAStar.

4.2.3. Isolation and manipulation of RNA

4.2.3.1. Isolation of RNA from *S. aurantiaca* cells

All solutions used in RNA preparation were treated with diethyl pyrocarbonate (DEPC) except Tris-containing buffer. DEPC was added to the solution to a final concentration of 0.1% and the solution was incubated at 37°C overnight with shaking and subsequently autoclaved. Tris-containing buffer was prepared with DEPC-treated H_2O. Undiscardable tubes, glassware and plastic materials were submerged in DEPC-treated H_2O overnight and then rinsed with ethanol.

S. aurantiaca was grown under appropriate conditions. 40 ml of log phase cells was transferred to a centrifuge tube. Ice was added to the tube to cool down the cell culture rapidly. After centrifugation at 5,000 × g for 15 min at 4°C, the cell pellet was resuspened in 250 µl of cold sucrose buffer in an eppendorf tube. 125 µl of lysis buffer was added (vortex vigorously) and the tube was incubated at 65°C for 90 seconds. 250 µl of H_2O-saturated phenol was added to the tube and the mixture was vortexed. After incubating at 65°C for 3 min, the tube was transferred to -70°C (methanol + solid C_2O) for 30 seconds. After centrifugation at 13,000 rpm for 10 min at room temperature, the supernatant was extracted with acidic phenol for twice. RNA was precipitated in 1 ml of ethanol containing 40 µl of 3 M NaAc (pH 4.5) at -20°C for 2.5 hrs. The RNA pellet was washed once with 70 % ethanol, air–dried and resuspended in 180 µl of RNA-storage buffer. 20 µl of 10 × DNase buffer and 10 units of DNase (RNase free) were added to the RNA solution to digest the DNA. The mixture was incubated at room temperature for 30 min. 20 µl of 0.2 M EDTA solution (pH 7.0) was added to stop the reaction. The solution was extracted twice with Tris-saturated phenol, once with phenol/chloroform, and once with chloroform. RNA was precipitated again in 1 ml of ethanol containing 25 µl of 3M NaAc (pH 7.0) at -20°C for 3

hrs. After centrifugation at 13,000 rpm for 15 min at 4°C, the RNA pellet was washed once with 70% ethanol, air dried, and dissolved in 40 µl of RNA storage buffer. The concentration of RNA was determined by spectrometry. For pure RNA, the ratio of OD_{260} to OD_{280} should be 2.0.

1 OD_{260} = 40 µg/ml RNA.

Sucrose buffer: 0.3 M sucrose, 10 mM NaAc, pH 4.5

Lysis buffer: 2% SDS, 10 mM NaAc, pH 4.5

RNA storage buffer: 20 mM Na-phosphate buffer, pH 6.5, 1 mM EDTA

10 × DNase buffer: 0.2 M NaAc, pH 4.5, 0.1 M $MgCl_2$, 0.1 M NaCl

4.2.3.2. RNA electrophoresis

RNA electrophoresis was performed on a 1% agarose-formaldehyde gel. One gram agarose was melted in 72 ml DEPC-treated H_2O and then incubated in a 60°C water bath. 18 ml of formaldehyde (37%) and 10 ml of 10 × MOPS buffer were added to the agarose suspension and mixed with it. The gel was poured in a hood exactly one hour before electrophoresis. 5 µg RNA was mixed with 3 volumes of denaturing buffer. The sample was incubated in a 60°C water bath for 10 min, then chilled down immediately on ice. 1/10 volume of RNA loading buffer was mixed with the sample before it was loaded onto the gel. Electrophoresis was carried out in 1 × MOPS buffer in a hood at 120 V for 10 min and then at 25 V for about 13 hours with buffer circulation. The gel was stained with 0.5 µg/ml ethidium bromide solution for 30 min and destained in H_2O for one hour or longer. RNA bands were visualized on a transilluminator (254 nm) and recorded with a video copy processor.

10 × MOPS buffer: 0.2 M MPOS, 80 mM NaAc, 10 mM EDTA, pH 7.0

Denaturing buffer: 64% formamide, 23% formaldehyde (7.9 M), 26 mM MOPS

10 × RNA loading buffer: 50% glycerol, 1 mM EDTA, pH 8.0, 0.4% bromphenolblue, 0.4% xylence cyanole

4.2.3.3. Northern transfer and hybridisation

After RNA electrophoresis, the gel was incubated in 0.05 M NaOH, 0.15 M NaCl buffer for 30 min, then in 0.1 M Tris (pH 7.5), 0.15 M NaCl buffer for 30 min. The gel was rinsed twice with DEPC-H_2O. RNA was transferred to a Biodyne B nylon membrane with a vacuum blotter in 10 × SSC buffer for 90 min at 60 mbar. The membrane was treated with UV cross-linking when the membrane was still wet. The membrane was rinsed twice with 2 × SSC buffer. Prehybridisation was carried out at 68°C for 4 hrs in perhybridisation buffer. Hybridisation was performed by addition of ^{32}P-labelled DNA probe to the prehybridisation buffer at 68°C overnight. The membrane was washed twice with 2 × SSC, 0.1% SDS buffer for 10 min at room temperature and twice with 0.2 × SSC, 0.1% SDS buffer for 15 min at 68°C. The membrane was then autoradiographed.

Prehybridisation buffer: 6 × SSC, 2 × Denhardt's, 0.1% SDS,
0.1 µg/ml denatured salmon sperm DNA

4.2.3.4. Reverse transcription polymerase chain reaction (RT- PCR)

Messenger RNA was transcribed by AMV-reverse transcriptase (AMV-RT) using an appropriate primer to produce ssDNA. The standard reactions were as follows:

Primer (3' → 5'): 100 pmol

Total RNA:	1-3 µg

DEPC-H_2O was added to a final volume of 10 µl

The reaction mixture was incubated at 70°C for 5 min and then cooled down to room temperature.

5 × AMV-RT buffer:	5 µl
dNTP (10 mM for each in DEPC-H_2O):	2.5 µl
RNasin Ribonuclease Inhibitor:	25 units
Sodium pyrophosphate (40 mM, prewarm at 42°C):	2.5 µl
AMV-RT:	15 units

DEPC-H_2O was added to a final volume of 25 µl

After incubation at 42°C for one hr, the reaction was stopped by addition of 25 µl of 0.1 M NaOH, 4 mM EDTA buffer. The solution was heated up to 65°C for 20 min. 5 µl of this solution was used as a template to amplify the target gene by normal PCR procedure.

4.2.3.5. Primer extension

The transcription initiation site of a target gene was determined by primer extension. The reverse primer was designed based on the result of Northern hybridisation. The size of the mRNA of the target gene should be roughly known. The reverse primer should be complementary to the sequence near the transcriptional start site. The standard reactions were as follows:

I. Hybridisation:

Total RNA:	10-100 µg
^{32}P-labelled primer:	1 pmol (2×10^6 cpm/pmol)
10 × hybridisation buffer:	1.5 µl
DEPC-H_2O:	to 15 µl

The reaction was incubated at 65°C for 90 min.

II. cDNA synthesis:

Solution from I:	15 µl
5 × AMV-RT buffer:	9 µl
10 × dNTP mixture (2.5 mM for each):	4.5 µl
DEPC-H_2O:	to 45 µl
AMV-RT:	15 units

The reaction mixture was incubated at 42°C for one hr.

III. Digestion of RNA:

Solution from II:	45 µl
RNase mix:	105 µl

The reaction mixture was incubated at 37°C for 15 min. 15 µl of 3 M NaAc (pH 4.5) was added to the reaction and cDNA was extracted once with 150 µl of phenol/chloroform. Then 15 µl of 3 M NaAc (pH 4.5) was added and the cDNA was precipitated with 3 volumes of ethanol at -20°C for 3 hrs. The cDNA pellet was washed once with 70% ethanol, dried under vacuum and dissolved in 3 µl of TE buffer. 3 µl of stop buffer (from Sequenase version 2.0 DNA sequencing kit, Amersham) was added to the solution and the synthesised cDNA

was analysed on a sequence gel: 6% polyacrylamide containing 7 M urea. Normal double-stranded DNA sequence reaction of this investigated region was performed on the same gel as molecular marker.

10 × hybridisation buffer:	1.5 M KCl, 0.1 M Tris, pH 8.3, 10 mM EDTA
RNase mix:	20 µg/ml RNase A
	100 µg/ml denatured salmon sperm DNA

4.2.4. Protein purification and analysis

4.2.4.1. Protein electrophoresis techniques

I. SDS containing polyacrylamide gel electrophoresis (SDS-PAGE)

The cell pellet from 1 ml culture of *E. coli* or 5 ml of *S. aurantiaca* (1.6×10^8 cells/ml) was resuspended in 100 µl of H_2O and mixed with 100 µl of 2 × Protein loading buffer and heated up to 95°C for 5 min. After chilling down on ice, protein samples were separated on 8% to 15% SDS–polyacrylamide separating gel with a 3% stacking gel (Laemmli, 1970). After electrophoresis in 1 × SDS-Tris-Glycin buffer at 25 mA, the gel was stained with Coomassie Brilliant Blue R250 or with Silver. If the gel would be used for immunoblot, the transfer was carried out immediately without staining.

Solutions for SDS-PAGE:

Stacking gel:

Tris-HCl (pH6.8)	125 mM
Acrylamide:bis-acrylamide	3% (30:0.8)
SDS	0.1
APS	0.4%
TEMED	0.4%

Separating gel:

Tris-HCl (pH 8.8)	375 mM
Acrylamide: bis-acrylamide	8-15% (30:0.8)
SDS	0.1
APS	0.5%
TEMED	0.25%

10 × SDS-Tris-Glycin buffer:

Tris-HCl (pH 6.8)	330 mM
Glycine	1.90 M
SDS	1%

2 × Protein loading buffer:

Tris-HCl (pH 6.8)	100 mM
DTT	200 mM
SDS	4%
Bromophenol blue	0.2%
Glycerol	20%

II. Coomassie blue staining

After electrophoresis, the polyacrylamide gel was stained in staining solution for 20 min at room temperature with gentle shaking. Then the gel was destained in destaining solution for one to four hrs. The destaining solution was changed at least twice.

Staining solution:

Methanol	45%
Acetic acid	10%
Coomassie brilliant blue R250	0.2%

Destaining solution:

Methanol	5%
Acetic acid	4.2%

III. Silver staining

After electrophoresis, the polyacrylamide gel was fixed in solution A at room temperature for one hr with gentle shaking. The gel was then rinsed thrice with 50% ethanol, each for 10 min, and then rinsed thrice with H_2O, each for 20 seconds. The gel was transferred into solution B for one min, then rinsed thrice with H_2O, each for 20 seconds. After staining in solution C for 20 min with gentle shaking, the gel was rinsed with H_2O. Finally, the gel was developed in solution D until enough signals were obtained. The gel was transferred into solution A to stop the reaction.

Solution A:

Methanol	50%
Acetic acid	12%
Formaldehyde	0.05%

Solution B:

Na-thiosulphate	0.8 mM

Solution C:

$AgNO_3$	12 mM
Formaldehyde	0.075%

Solution D:

Na_2CO_3	140 mM
Na-thiosulphate	0.024 mM
Formaldehyde	0.05%

4.2.4.2. Western blotting and hybridisation

After electrophoresis, proteins were transferred from the gel to a nitrocellulose filter in a Transblot Cell between six sheets of Transfer buffer-saturated Whatman papers (Sambrook *et al.*, 1989). The transfer was performed under 0.8 mA/cm^2 for one hr at 4°C with cooling water recycling. The transferred protein on the membrane were reversibly visualized with 0.2% Ponceau S.

The nitrocellulose membrane bound protein was first incubated with TBST buffer containing 5% milk powder at room temperature for one hr to block unspecific binding. The membrane was then incubated with a specific antibody, 1:2000 diluted in TBST buffer, at room temperature for one hr or at 4°C overnight with gentle shaking. After washing twice with TBST buffer at room temperature for 10 min, the membrane was incubated

with anti-rabbit IgG (alkaline phosphatase conjugated), 1:5000 diluted in TBST buffer, at room temperature for one hr with gentle shaking. After washing twice with TBST buffer for 15 min, the membrane was developed in AP buffer containing 0.67% NBT solution and 0.33% BCIP solution until enough signals were obtained. The reaction was stopped by transferring the membrane into a 50 mM EDTA solution, pH8.0.

Transfer buffer

Tris-HCl, pH 8.5	25 mM
Glycine	150 mM
Methanol	10 %

TBST buffer

Tris-HCl, pH 8.0	0.1 M
NaCl	0.15 M
NP40	0.5%

AP buffer

Tris-HCl, pH 9.5	0.1 M
EDTA	5 mM
$MgCl_2$	5 mM

NBT buffer

Nitroblue tetrazoliumchloride	50 mg/ml in 70% dimethylformamide

BCIP buffer

5-bromo-4-chloro-3-indolyl-phosphate	50 mg/ml in 100% ethanol

4.2.4.3. Determination of protein concentration

I. Bradford assay

The Protein assay kit (BioRAD) was used for the determination of 1-20 μg protein (concentration <10 mg/ml) using BSA as a standard protein (Bradford, 1976).

0.1 ml of protein solution was mixed with 2.0 ml of working reagent and incubated at 37°C for 30 min. After incubation, the tube was chilled down to room temperature and the absorbance of the protein solution was measured at 562 nm. The standard curve was made by measuring a BSA solution of 0-1.2 mg/ml at OD_{562}. The concentration of the unknown protein was calculated according to the standard curve.

II. Absorbance

When the amino acid sequence of certain protein is known, the concentration of the protein was determined by the absorbance according to the Lambert-Beer law: $A = \varepsilon \times c \times l$, where A is the absorbance, c is the molar concentration, l is the pathlength in cm and ε is the total molar absorbance coefficient. The absorption of the protein in the range of 230-300 nm was determined by the aromatic side chains of tyrosine, tryptophan and phenylalanine:

	Absorbance	
Compound	λ_{max} (nm)	ε_{max} (M^{-1}, cm^{-1})
Tryptophan	280	5600
Tyrosine	274	1400
Phenylalanine	257	200

4.2.4.4. Purification of overexpressed protein from *E. coli* cells

I. Denaturing purification of insoluble proteins

When the *hspA* gene was cloned in vector pQE9 and overexpressed in *E. coli* strain M15, the 6 × His-tagged HspA fusion protein was purified under denaturing conditions:

Cells containing the heterologously expressed protein from 500 ml culture were harvested by centrifugation at 4,000 × g for 20 min and resuspended in buffer A at the ratio of 5 ml A buffer per gram wet weight of cells. The cell suspension was stirred for one hr at room temperature and centrifuged at 10,000 × g for 15 min at 4°C. The supernatant was collected. 8 ml of 50% slurry of Ni-NTA-agarose pre-equilibrated with buffer A was added to the supernatant. The mixture was stirred at room temperature for 45 min and then loaded into a column with 1.6 cm diameter. The column was washed with 10 volumes of buffer A at a flow rate of 10-15 ml/hr, followed with 5 volumes of buffer B until the A_{280} of flow-through was < 0.01. The column was then washed with buffer C until the A_{280} of flow-through was < 0.01. The recombinant protein was eluted with 10-20 ml of buffer D, followed with 10-20 ml of buffer E. 3 ml fractions were collected and analysed by SDS-PAGE. The column was then washed with 20 ml of buffer F. 3 ml fractions were collected and analysed by SDS-PAGE. Generally, monomers were eluted in buffer D, while multimers, aggregates and protein with two 6 ×⊇His-tags would be eluted in buffer E. The fractions contained the target protein were then transferred into a pre-treated dialyse tubing (Sambrook *et al*., 1989) and dialysed in 100 volumes of 1 × Dialyse buffer at 4°C overnight. The protein was concentrated by filtration using Centriprep 10 with the recommended protocol of the manufacturer. The protein was stored at -80°C.

Buffer A:	6 M GuHCl, 0.1 M Na-phosphate, 0.01 M Tris-Cl, pH 8.0
Buffer B:	8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-Cl, pH 8.0
Buffer C:	8 M urea, 0 1 M Na-phosphate, 0.01 M Tris-Cl, pH 6.3
Buffer D:	8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-Cl, pH 5.9
Buffer E:	8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-Cl, pH 4.5
Buffer F:	6 M GuHCl, 0.2 M acetic acid
10 × Dialyze buffer:	0.4 M HEPES-KOH, pH 7.5, 0.5 M KCl, 5 mM $MgCl_2$, 10 mM EDTA, 10% glycerol

II. Native purification of cytoplasmic proteins

When the *hspA* gene was cloned in vector pQE9 and overexpressed in *E. coli* strain GI698, the 6 × His-tagged HspA fusion protein was purified under native conditions:

Cells from one litre culture were harvested by centrifugation at 4,000 × g for 20 min and resuspended in Sonication buffer at a ratio of 5 ml buffer per gram wet weight of cells. The sample was then frozen in dry ice/ethanol and thawed in a cold water bath. The cells were disintegrated by sonication on ice (one min burst/one min cooling, 50% output) by Branson Sonifier B-15. The cell breakage was monitored by measuring the nucleic acid release at A_{260} of the flow-through. After centrifugation at 10,000 × g for 20 min at 4°C, the supernatant was mixed with 8 ml of 50% slurry of Ni-NTA-agarose pre-equilibrated with sonication buffer. The mixture was stirred on ice for one hr. The resin was then loaded into a column with 1.6 cm diameter and washed with sonication buffer at a flow rate of 0.5 ml/min until the A_{280} of flow-through was < 0.01. The column was then washed with Wash buffer until the A_{280} of flow-through was < 0.01. The protein was eluted with 30 ml of a 0-0.5

M imidazole gradient in Wash buffer. 2 ml fractions were collected and analysed on SDS-PAGE, but instead of heating the samples up to 95°C, the samples were mixed with an equal volume of 2 × Protein loaded buffer and incubated at 37°C for 10 min before loading onto the gel. Target protein was dialysed and concentrated and stored as described above.

Sonication buffer: 50 mM Na-phosphate pH 8.0, 300 mM NaCl, 1 mM PMSF

Wash buffer: 50 mM Na-phosphate pH 6.0, 300 mM NaCl, 10% Glycerol, 1 mM PMSF

III. Regeneration of Ni-superagarose resin

The resin of Ni-NTA-agarose can be reused for 3 to 5 times to purify the same protein. The washing procedure was as follows:

Wash the column with 2 volumes of 0.2 M acetate acid.

Wash the column with 2 volumes of 0.05 M EDTA, pH 8.0.

Wash the column with 2 volumes of 0.1 M $NiSO_4$ $6H_2O$.

Wash the column with 2 volumes of 0.2 M acetate acid.

Wash the column with 4 volumes of H_2O.

Wash the column with 2 volumes of 30% ethanol and keep the resin in it.

Before using, the column would be equilibrated with 2 volumes of buffer A (denaturing condition) or Sonication buffer (native condition).

4.2.4.5. Determination of protein oligomer by HPLC size exclusion chromatography (SEC)

SEC was performed using a TosoHaas TSK G4000 SW column. Chromatography was carried out using 100 mM HEPES soultion, pH 7.5, with a flow rate of 0.5 ml/min and a sample size of 100 µl. The native protein sample was pre-dialysed in 40 mM HEPES buffer, pH 7.5 and centrifuged for 10 min at 14,000 × g before application. The concentration of the protein was determined by measuring the absorption of the protein at the wavelength ranging from 230nm to 300 nm. The elution of the protein sample was detected by fluorescence at an excitation wavelength of 280 nm and an emission wavelength of 330 nm using a Merck Hitachi fluorescence detector.

4.2.4.6. Determination of ß-galactosidase activity

30 ml of vegetative or heat shocked or indole treated *S. aurantiaca* cells were harvested by centrifugation at 4,000 × g for 15 min at 4°C. The cell pellet was washed once with MOPS buffer (50 mM MOPS, pH 7.5, 10 mM $MgCl_2$) and resuspended in 150 µl of the same buffer. The cells were broken by sonication at 4°C with a Branson Sonifier B-15 (one min burst/one min cooling, 50% output). After centrifugation at 13,000 rpm for 30 min at 4°C with an eppendorf centrifuge, the protein concentration in the supernatant was measured by Bradford assay.

ß-galactosidase activity was measured by using 4-MUG as a substrate, which is hydrolysed by ß–galactosidase to yield the highly fluorescent methylumbelliferone.

A 10 µg protein sample was diluted in 100 µl of degassed MOPS buffer containing 1 mM PMSF and 1 mM DTT. Then, 300 µl of degassed buffer A was added to the protein sample buffer and the solution was incubated at 37°C for 30 min. The reaction was stopped by addition of 3 ml of degassed 0.1 M Glycine solution, pH 10.3.

The fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 450 nm using a Shimadzu RF 5000 fluorescence spectrophotometer.

Buffer A: 10 mM Na-phosphate, pH 7.0, 0.1 M NaCl, 1 mM $MgCl_2$,
10 µg 4-MUG/300 µl, 0.1% BSA

4.2.4.7. Measurement of light scattering

Citrate synthase (15 µM) was denatured in a buffer of 6.6 M GuHCl, 20 mM DTT, 40 mM HEPES, pH 7.5 at room temperature for 2 hrs. The mixture was diluted 1:100 in 40 mM HEPES buffer, pH 7.5 at 25 °C, in the presence and absence of $HspA_{His}$ or other proteins (see result part). To monitor the kinetics of aggregation, light scattering was measured with Perkin Elmer MPF44A fluorescence spectrophotometer in stirred and thermostatted quartz cuvettes. Both the excitation and emission wavelengths were 500 nm with a spectral bandwidth of 2 nm.

Aggregation of insulin B chain was monitored by measuring the apparent absorption due to a scattering at 400 nm. Insulin dissolved in a buffer of 40 mM Na-phophate, pH 7.0, 100 mM NaCl, 2 mM EDTA to a concentration of 0.35 mg/ml was reduced with 50 mM DTT in the presence and absence of $HspA_{His}$ or other proteins (see result part).

4.2.4.8. Determination of citrate synthase activity

The activity of citrate synthase (CS) was determined by the appearance of free SH group of the released CoASH: Acetyl-CoA + oxaloacetate^{2-} + H_2O ↔ citrate^{2-} + CoASH + H^+ (Srere, 1969).

1 ml working buffer was incubated at 25°C in a cuvette. 4 µl native or denatured CS (15 µM) was diluted 1:100 in 50 mM Tris-Cl buffer, pH 8.0 in the presence and absence of $HspA_{His}$ or other agents (see result part) with stirring at 25°C. The time was accounted after starting the dilution. 20 µl of diluted CS was added after different time periods to the cuvette containing working buffer. The absorbance of the reaction was measured at 412 nm for one min to calculate the CS activity.

Working buffer (1 ml): 930 µl of 50 mM Tris-Cl
10 µl of 10 mM oxaloacetate (in 50 mM Tris-Cl, pH 8.0)
10 µl of 1 mM 5, 5'-dithiobis-(2-nitrobenzoate) (DTNB)
(in 50 mM Tris-Cl, pH 8.0)
30 µl of 10 mM of acetyl-CoA (in 50 mM Tris-Cl, pH 8.0)

4.2.5. Protein-DNA interaction assay

4.2.5.1. Gel retardation assay

20 ml of vegetative or heat shocked or indole induced *S. aurantiaca* cells were harvested by centrifugation at 4,000 × g for 15 min at 4°C. After washing twice with 5 mM HEPES, pH 7.2, 0.5 mM $CaCl_2$ buffer, the cell pellet was resuspended in 400 µl of cell extract buffer. The cells were broken by sonication using a Branson Sonifier B-15 (one min burst/one min cooling, 50% output) at 4°C. After centrifugation at 13,000 rpm for 30 min at 4°C with an eppendorf centrifuge, the protein concentration in the supernatant was measured by Bradford assay.

The target DNA fragment was labelled by γ^{32}P-ATP. The signal of labelled DNA was measured with a ß-counter (Tri-carb 1500).

The protein-DNA interaction reaction was as follows:

4 × binding buffer:	5 µl
Poly d (I-C) (0.33mg/ml in TE):	2 µl
Cell extract:	5 µg
^{32}P-labelled DNA:	30,000 cpm
H_2O	up to 20 µl

*If special DNA was used as competitor in the reaction (see result part), the amount of the competitive DNA was in a 200 fold molar excess.

The reaction mixture was incubated at room temperature for 5 min before addition of the labelled DNA to the reaction. After addition of the labelled DNA, the reaction was incubated at room temperature for further 10 min and then loaded onto a 4.2% polyacrylamide gel in 0.5 × TBE buffer and the electrophoresis was performed in the same buffer under 3 V/cm at room temperature. After electrophoresis, the gel was dried between Whatman 3MM papers under vacuum and then autoradiographed.

Cell extract buffer:	20 mM HEPES, pH 7.9
	25% Glycerol
	0.1 M $NaCl_2$
	1.5 mM $MgCl_2$
	1 mM PMSF
	1 mM DTT
4 × Binding buffer:	50 mM KCl
	80 mM HEPES, pH 7.9
	4 mM EDTA
	4 mM DTT
	16% Ficoll

4.2.5.2. Phosphorylation of cell extracts

To analyse whether phosphorylation is involved in the protein-DNA interaction, 5 µg of cell extracts was incubated with 50 mM acetyl phosphate at 37°C for one hr before carrying out the protein-DNA interaction reaction (4.5.2.1).

V. Summary

HspA (originally designated SP21) of *S. aurantiaca* has been isolated from indole induced spores of the *S. aurantiaca* DW4/3-1 strain. Using specific antisera, HspA was also detected in fruiting body derived spores, in heat shocked cells, and in oxygen deprivation cells, but not in vegetative cells growing under normal conditions (Heidelbach *et al*., 1993b).

Immunoelectron microscopy has revealed that HspA is located mainly at the cell periphery in heat shocked cells and either at the cell periphery or within the cytoplasm in indole treated cells, often in a cluster form. In fruiting body derived spores, HspA was located mainly at the cell wall, preferentially at the outer periphery. Furthermore, HspA was found also to be associated with cellular remnants within the stalk and within the peripheral horizon next to the fruiting body (Lünsdorf *et al*., 1995).

The corresponding gene of HspA (*hspA*) was first isolated from a gene expression library in λgt11 with antisera against HspA. The deduced amino acids sequence of *hspA* shows homology to other small heat shock proteins of plants, suggesting that it is a member of the small heat shock protein family (Heidelbach *et al*., 1993a).

A *Pst*I fragment harbouring the *hspA* gene and its correct flanking regions was isolated from *S. aurantiaca* DW4/3-1 genome. The ORF of *hspA* that is 510 bp long was corrected by sequence analysis. It encodes a protein composed of 169 amino acids with an estimated molecular mass of 19357.66 Da. The α-crystallin domain is conserved in HspA. Besides this, a new ORF, *ephA*, was found downstream of *hspA* in the opposite orientation and 66 bp apart from *hspA*, which is 1.323 kbp long and encodes a polypeptide composed of 440 amino acids. The deduced amino acid sequence of this ORF has a significant similarity with microsomal epoxide hydrolase from mammals. An unknown ORF, *orfX*, was found upstream of *hspA* in the opposite orientation and 256 bp apart from *hspA*, which is 456 bp long and encodes a polypeptide of 151 amino acids. No similarity was found between the deduced amino acid sequence of *orfX* product and known proteins.

The monocistronic structure of *hspA* was determined by Northern analysis. Two transcripts of *hspA* were observed in response to heat stimulation. One is about 650 nts, the other is about 700 nts in length. However, only the 650 nts transcript of *hspA* appeared during indole treatment. A unique transcription initiation site of *hspA* was determined by primer extension after heat shock and indole treatment that is located at bp -50 relative to the translation start site of *hspA* assigned +1.

The promoter activity of *hspA* was studied either after integration of the promoter into the *attB* site or to the *hspA* locus using a promoterless *rtrpA-lacZ* gene as the reporter gene. The expression level and the time dependent expression of the *lacZ* gene were the same at both sites.

Although the transcription of *hspA* starts at the same site under heat shock conditions and during indole treatment, the promoter mapping assays revealed that different promoter regions of *hspA* are required under the two conditions. The promoter region of *hspA* needed for heat shock dependent maximum expression extends up to bp -225. For the indole dependent maximum activation of the *hspA* promoter (P*hspA*), 587 bp upstream sequence of *hspA* is required. At least, three regulatory regions are involved in the transcriptional regulation of *hspA* under heat shock conditions. The first region spans bp -85 to bp -56 that contains the RNA polymerase binding site. Deletion of this region completely blocks the activity of P*hspA*. The second region ranges from bp -223 to bp -141 that carries the putative transcription enhancer binding sites. Heat shock and phosphorylation increase the binding of the putative enhancer(s) to P*hspA*. Deletion of the second region reduces the activity of P*hspA* by about half. Finally, a deletion of the region from bp -140 to bp -86 together with the second region abolished the P*hspA* activity, suggesting that a *cis*-acting element exists just upstream to the -35 region. Taken together, the transcription of *hspA* seems to be mainly positively regulated in *S. aurantiaca* under heat shock conditions.

Since HspA is an abundant small heat shock protein in *S. aurantiaca* cells, it was speculated that the protein plays a role in the cell thermotolerance and cell differentiation of *S. aurantiaca*. A *hspA* deletion mutant (SH1) was constructed. However, vegetative cell cycle and developmental cell cycle including aggregation, stalk formation, fruiting body formation, sporulation and germination, and the ultrastructure of spores of SH1 did not shown any difference as compared to those of the wild-type strain DW4/3-1.

The chaperone properties of the recombinant *hspA* with 6 His-tag ($HspA_{His}$) were revealed by *in vitro* biochemical assays. The $HspA_{His}$ protein tended to assemble into a large complex that consists of 26 subunits with a molecular mass of 560 kDa as judged by SEC. This oligomer of $HspA_{His}$ is able to interact with unfolded citrate snythase (CS) and prevents its precipitation from solution. But it is not able to prevent the precipitation of the unfolded B–chain of insulin. A stable complex may thus formed between $HspA_{His}$ and the unfolded CS because the unfolded CS can not dissociate from the complex. Hence, $HspA_{His}$ alone is unable to facilitate the refolding procedure of unfolded CS.

VI. References

Allen, S.P., Polazzi, J.O., Gierse, J.K. and Easton, A.M. (1992) Two novel heat shock genes encoding proteins produced in response to heterologous protein expression in *Escherichia coli*. *J. Bacteriol*, **174**, 6938-6947.

Amin, J., Ananthan, J. and Voellmy, R. (1988) Key features of heat shock regulatory elements. *Mol. Cell. Biol*, **8**, 3761-3769.

Aoyama, A., Frohli, E., Schafer, R. and Klemenz, R. (1993) Alpha B-crystallin expression in mouse NIH 3T3 fibroblasts: glucocorticoid responsiveness and involvement in thermal protection. *Mol. Cell. Biol*, **13**, 1824-1835.

Apelian, D. and Inouye, S. (1990) Development-specific sigma-factor essential for late-stage differentiation of *Myxococcus xanthus*. *Genes & Dev*, **4**, 1396-1403.

Apelian, D. and Inouye, S. (1993) A new putative sigma factor of *Myxococcus xanthus*. *J. Bacteriol*, **175**, 3335-3342.

Arand, M., Grant, D.F., Beetham, J.K., Friedberg, T., Oesch, F. and Hammock, B.D. (1994) Sequence similarity of mammalian epoxide hydrolases to the bacterial haloalkane dehalogenase and other related proteins. Implication for the potential catalytic mechanism of enzymatic epoxide hydrolysis. *FEBS Lett*, **338**, 251-256.

Arand, M., Wagner, H. and Oesch, F. (1996) Asp333, Asp495, and His523 form the catalytic triad of rat soluble epoxide hydrolase. *J. Biol. Chem*, **271**, 4223-4229.

Arata, S., Hamaguchi, S. and Nose, K. (1997) Inhibition of colony formation of NIH 3T3 cells by the expression of the small molecular weight heat shock protein HSP27: involvement of its phosphorylation and aggregation at the Dmterminal region. *J. Cell. Physiol*, **170**, 19-26.

Arnold, J.W. and Shimkets, L.J. (1988) Cell surface properties correlated with cohesion in *Myxococcus xanthus*. *J. Bacteriol*, **170**, 5771-5777.

Arrigo, A.-P. and Landry, J. (1994) Expression and function of the low-molecular-weight heat shock proteins. In Morimoto, R.I., Tissieres, A. and Georgopoulos, C. (eds.), *The biology of heat shock proteins and molecular chaperones*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 335-374.

Arrigo, A.P., Suhan, J.P. and Welch, W.J. (1988) Dynamic changes in the structure and intracellular locale of the mammalian low-molecular-weight heat shock protein. *Mol. Cell. Biol*, **8**, 5059-5071.

Arrigo, A.P. and Welch, W.J. (1987) Characterization and purification of the small 28,000-dalton mammalian heat shock protein. *J. Biol. Chem*, **262**, 15359-15369.

Atkinson, B.G., Raizada, M., Bouchard, R.A., Frappier, R.H. and Walden, D.B. (1993) The independent stage-specific expression of the 18-kDa heat shock protein genes during microsporogenesis in Zea mays L. *Dev. Genet*, **14**, 15-26.

Avedissian, M. and Lopes Gomes, S. (1996) Expression of the *groESL* operon is cell-cycle controlled in *Caulobacter crescentus*. *Mol. Microbiol*, **19**, 79-89.

Babst, M., Hennecke, H. and Fischer, H.M. (1996) Two different mechanisms are involved in the heat-shock regulation of chaperonin gene expression in *Bradyrhizobium japonicum*. *Mol. Microbiol*, **19**, 827-839.

Baler, R., Dahl, G. and Voellmy, R. (1993) Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. *Mol. Cell. Biol*, **13**, 2486-2496.

Beetham, J.K., Grant, D., Arand, M., Garbarino, J., Kiyosue, T., Pinot, F., Oesch, F., Belknap, W.R., Shinozaki, K. and Hammock, B.D. (1995) Gene evolution of epoxide hydrolases and recommended nomenclature. *DNA. Cell. Biol*, **14**, 61-71.

Bell, P.A. and Kasper, C.B. (1993) Expression of rat microsomal epoxide hydrolase in *Escherichia coli.* Identification of a histidyl residue essential for catalysis. *J. Biol. Chem*, **268**, 14011-14017.

Benaissa, M., Vieyres Lubochinsky, J., Odeide, R. and Lubochinsky, B. (1994) Stimulation of inositide degradation in clumping *Stigmatella aurantiaca*. *J. Bacteriol*, **176**, 1390-1393.

Biran, D. and Kroos, L. (1997) *In vitro* transcription of *Myxococcus xanthus* genes with RNA polymerase containing sigmaA, the major sigma factor in growing cells. *Mol. Microbiol*, **25**, 463-472.

Bond, U. and Schlesinger, M.J. (1987) Heat-shock proteins and development. *Adv. Genet*, **24**, 1-29.

Bowden, M.G. and Kaplan, H.B. (1998) The *Myxococcus xanthus* Lipopolysaccharide O-antigen is required for social motility and multicellular development. *Mol. Microbiol*, **30**, 275-284.

Bowman, W.C. and Kranz, R.G. (1998) A bacterial ATP-dependent, enhancer binding protein that activates the housekeeping RNA polymerase. *Genes & Dev.*, 1884-1893.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*, **72**, 248-254.

Brandner, J.P. and Kroos, L. (1998) Identification of the omega4400 regulatory region, a developmental promoter of *Myxococcus xanthus*. *J. Bacteriol*, **180**, 1995-2004.

Bucca, G., Ferina, G., Puglia, A.M. and Smith, C.P. (1995) The *dnaK* operon of *Streptomyces coelicolor* encodes a novel heat-shock protein which binds to the promoter region of the operon. *Mol. Microbiol*, **17**, 663-674.

Bucca, G., Hindle, Z. and Smith, C.P. (1997) Regulation of the dnaK operon of *Streptomyces coelicolor* A3(2) is governed by HspR, an autoregulatory repressor protein. *J. Bacteriol*, **179**, 5999-6004.

Bukau, B. (ed.) (1999) *Molecular chaperones and folding catalysts.* Harwood academic publishers, Amsterdam.

Caspers, G.J., Leunissen, J.A. and de Jong, W.W. (1995) The expanding small heat-shock protein family, and structure predictions of the conserved "alpha-crystallin domain". *J. Mol. Evol*, **40**, 238-248.

Chang, B.Y. and Dworkin, M. (1996) Mutants of *Myxococcus xanthus dsp* defective in fibril binding. *J. Bacteriol*, **178**, 697-700.

Chang, Z., Primm, T.P., Jakana, J., Lee, I.H., Serysheva, I., Chiu, W., Gilbert, H.F. and Quiocho, F.A. (1996) *Mycobacterium tuberculosis* 16-kDa antigen (Hsp16.3) functions as an oligomeric structure *in vitro* to suppress thermal aggregation. *J. Biol. Chem*, **271**, 7218-7223.

Chen, H., Keseler, I.M. and Shimkets, L.J. (1990) Genome size of *Myxococcus xanthus* determined by pulsed-field gel electrophoresis. *J. Bacteriol*, **172**, 4206-4213.

Cheng, Y. and Kaiser, D. (1989a) *dsg*, a gene required for cell-cell interaction early in *Myxococcus* development. *J. Bacteriol*, **171**, 3719-3726.

Cheng, Y. and Kaiser, D. (1989b) *dsg*, a gene required for *Myxococcus* development, is necessary for cell viability. *J. Bacteriol*, **171**, 3727-3731.

Cheng, Y.L., Kalman, L.V. and Kaiser, D. (1994) The *dsg* gene of *Myxococcus xanthus* encodes a protein similar to translation initiation factor IF3. *J. Bacteriol*, **176**, 1427-1433.

Ciocca, D.R., Oesterreich, S., Chamness, G.C., McGuire, W.L. and Fuqua, S.A. (1993) Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. *J. Natl. Cancer. Inst*, **85**, 1558-1570.

Colado-Vides, J., Magasanik, B. and Gralla, J.D. (1991) Control site location and transcriptional regulation in *Escherichia coli*. *Microbiol. Rev*, **55**, 371-394.

Collada, C., Gomez, L., Casado, R. and Aragoncillo, C. (1997) Purification and in vitro chaperone activity of a class I small heat-shock protein abundant in recalcitrant chestnut seeds. *Plant. Physiol*, **115**, 71-77.

Collier, N.C. and Schlesinger, M.J. (1986) The dynamic state of heat shock proteins in chicken embryo fibroblasts. *J. Cell. Biol*, **103**, 1495-1507.

Connolly, L., Yura, T. and Gross, C.A. (1999) Autoregulation of the heat shock response in Procaryotes. In Bukau, B. (ed.) *Molecular chaperones and folding catalysts*. Harwood academic publishers, Amsterdam, pp. 13-34.

Coudart, M.P. (1998) Independent patterns of expression of two alternative sigma factors, *sigB* and *sigC*, of the myxobacterium *Stigmatella aurantiaca* during development. *Mol. Biol. Rep*, **25**, 183-188.

Cox, J.S. and Walter, P. (1996) A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell*, **87**, 391-404.

de Jong, W.W., Caspers, G.J. and Leunissen, J.A. (1998) Genealogy of the alpha-crystallin small heat shock protein superfamily. *Int. J. Biol. Macromol*, **22**, 151-162.

de Jong, W.W., Leunissen, J.A., Leenen, P.J., Zweers, A. and Versteeg, M. (1988) Dogfish alpha-crystallin sequences. Comparison with small heat shock proteins and Schistosoma egg antigen. *J. Biol. Chem*, **263**, 5141-5149.

de Jong, W.W., Leunissen, J.A. and Voorter, C.E. (1993) Evolution of the alpha-crystallin/small heat-shock protein family. *Mol. Biol. Evol*, **10**, 103-126.

de Lorenzo, V., M., H., U., J. and N., T.K. (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol*, **175**, 7762-7770.

Downard, J., Ramaswamy, S.V. and Kil, K.S. (1993) Identification of *esg*, a genetic locus involved in cell-cell signaling during *Myxococcus xanthus* development. *J. Bacteriol*, **175**, 7762-7770.

Downard, J. and Toal, D. (1995) Branched-chain fatty acids: the case for a novel form of cell-cell signalling during *Myxococcus xanthus* development. *Mol. Microbiol*, **16**, 171-175.

Downard, J.S., Kim, S.H. and Kil, K.S. (1988) Localization of the *cis*-acting regulatory DNA sequences of the *Myxococcus xanthus tps* and *ops* genes. *J. Bacteriol*, **170**, 4931-4938.

Duchene, A.M., Kieser, H.M., Hopwood, D.A., Thompson, C.J. and Mazodier, P. (1994a) Characterization of two *groEL* genes in *Streptomyces coelicolor* A3(2). *Gene*, **144**, 97-101.

Duchene, A.M., Thompson, C.J. and Mazodier, P. (1994b) Transcriptional analysis of *groEL* genes in *Streptomyces coelicolor* A3(2). *Mol. Gen. Genet*, **245**, 61-68.

Dworkin, M. (1985) *Developmental biology of the bacteria*. The Benjamin/Cummings Publishing Company, Inc, California.

Dworkin, M. (1994) Increases in the intracellular concentration of glycerol during development in *Myxococcus xanthus* S. Courtney Frasch [corrected and republished in FEMS Microbiol Lett. 1994 Oct 1;122(3):321-5]. *FEMS Microbiol. Lett*, **120**, 369-373.

Dworkin, M. (1996) Recent advances in the social and developmental biology of the myxobacteria. *Microbiol. Rev*, **60**, 70-102.

Eckstein, F. and Gish, G. (1989) Phophorothioates in molecalar biology. *TIBS*, 97-100.

Ehrnsperger, M., Graber, S., Gaestel, M. and Buchner, J. (1997) Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *EMBO. J*, **16**, 221-229.

Eiiehauge, E., Norregaard-Madsen, M. and Sogaard-Andersen, L. (1998) The FruA signal transduction protein provides a checkpoint for the temporal co-ordination of intercellular signals in *Myxococcus xanthus* development. *Mol. Microbiol*, **30**, 807-817.

Estrem, S.T., Gaal, T., Ross, W. and Gourse, R.L. (1998) Identification of an UP element consensus sequence for bacterial promoters. *Proc. Natl. Acad. Sci. U. S. A*, **95**, 9761-9766.

Falany, C.N., McQuiddy, P. and Kasper, C.B. (1987) Structure and organization of the microsomal xenobiotic epoxide hydrolase gene. *J. Biol. Chem*, **262**, 5924-5930.

Farahbakhsh, Z.T., Huang, Q.L., Ding, L.L., Altenbach, C., Steinhoff, H.J., Horwitz, J. and Hubbell, W.L. (1995) Interaction of alpha-crystallin with spin-labeled peptides. *Biochemistry*, **34**, 509-516.

Farnsworth, P.N., Frauwirth, H., Groth Vasselli, B. and Singh, K. (1998) Refinement of 3D structure of bovine lens alpha A-crystallin. *Int. J. Biol. Macromol*, **22**, 175-185.

Farnsworth, P.N., Groth Vasselli, B., Greenfield, N.J. and Singh, K. (1997) Effects of temperature and concentration on bovine lens alpha-crystallin secondary structure: a circular dichroism spectroscopic study. *Int. J. Biol. Macromol*, **20**, 283-291.

Fernandes, M., Xiao, H. and Lis, J.T. (1994) Fine structure analyses of the *Drosophila* and *Saccharomyces* heat shock factor--heat shock element interactions. *Nucleic. Acids. Res*, **22**, 167-173.

Fink, A.L. and Goto, Y. (eds.) (1997) *Molecular chaperones in the life cycle of proteins*. Marcel Dekker Inc., New York.

Fisseha, M., Gloudemans, M., Gill, R.E. and Kroos, L. (1996) Characterization of the regulatory region of a cell interaction-dependent gene in *Myxococcus xanthus*. *J-Bacteriol*, **178**, 2539-2550.

Frasch, S.C. and Dworkin, M. (1996) Tyrosine phosphorylation in *Myxococcus xanthus*, a multicellular prokaryote. *J. Bacteriol*, **178**, 4084-4088.

Gernold, M., Knauf, U., Gaestel, M., Stahl, J. and Kloetzel, P.M. (1993) Development and tissue-specific distribution of mouse small heat shock protein hsp25. *Dev. Genet*, **14**, 103-111.

Gerth, K., Bedorf, N., Irschik, H., Hofle, G. and Reichenbach, H. (1994) The soraphens: a family of novel antifungal compounds from *Sorangium cellulosum* (Myxobacteria). I. Soraphen A1 alpha: fermentation, isolation, biological properties. *J. Antibiot. Tokyo*, **47**, 23-31.

Gill, R.E. and Cull, M.G. (1986) Control of developmental gene expression by cell-to-cell interactions in *Myxococcus xanthus*. *J. Bacteriol*, **168**, 341-347.

Gill, R.E., Karlok, M. and Benton, D. (1993) *Myxococcus xanthus* encodes an ATP-dependent protease which is required for developmental gene transcription and intercellular signaling. *J. Bacteriol*, **175**, 4538-4544.

Glomp, I., Saulnier, P., Guespin Michel, J. and Schairer, H.U. (1988) Transfer of IncP plasmids into *Stigmatella aurantiaca* leading to insertional mutants affected in spore development. *Mol. Gen. Genet*, **214**, 213-217.

Gollop, R., Inouye, M. and Inouye, S. (1991) Protein U, a late-developmental spore coat protein of *Myxococcus xanthus*, is a secretory protein. *J. Bacteriol*, **173**, 3597-3600.

Goudreau, P.N. and Stock, A.M. (1998) Signal transduction in bacteria: molecular mechanisms of stimulus-response coupling. *Current opinon in Microbiology*, **1**, 160-169.

Grimm, B., Ish Shalom, D., Even, D., Glaczinski, H., Ottersbach, P., Ohad, I. and Kloppstech, K. (1989) The nuclear-coded chloroplast 22-kDa heat-shock protein of *Chlamydomonas*. Evidence for translocation into the organelle without a processing step. *Eur. J. Biochem*, **182**, 539-546.

Groenen, P.J., Merck, K.B., de Jong, W.W. and Bloemendal, H. (1994) Structure and modifications of the junior chaperone alpha-crystallin. From lens transparency to molecular pathology. *Eur. J. Biochem*, **225**, 1-19.

Gross, D.S., English, K.E., Collins, K.W. and Lee, S.W. (1990) Genomic footprinting of the yeast HSP82 promoter reveals marked distortion of the DNA helix and constitutive occupancy of heat shock and TATA elements. *J. Mol. Biol*, **216**, 611-631.

Groth Vasselli, B., Kumosinski, T.F. and Farnsworth, P.N. (1995) Computer-generated model of the quaternary structure of alpha crystallin in the lens [letter]. *Exp. Eye. Res*, **61**, 249-253.

Gulati, P., Xu, D. and Kaplan, H.B. (1995) Identification of the minimum regulatory region of a *Myxococcus xanthus* A-signal-dependent developmental gene. *J. Bacteriol*, **177**, 4645-4651.

Gyorgyey, J., Gartner, A., Nemeth, K., Magyar, Z., Hirt, H., Heberle Bors, E. and Dudits, D. (1991) Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. *Plant. Mol. Biol*, **16**, 999-1007.

Hagen, D.C., Bretscher, A.P. and Kaiser, D. (1978) Synergism between morphogenetic mutants of *Myxococcus xanthus*. *Dev. Biol*, **64**, 284-296.

Haley, D.A., Horwitz, J. and Stewart, P.L. (1998) The small heat-shock protein, alphaB-crystallin, has a variable quaternary structure. *J. Mol. Biol*, **277**, 27-35.

Hanlon, W.A., Inouye, M. and Inouye, S. (1997) Pkn9, a Ser/Thr protein kinase involved in the development of *Myxococcus xanthus*. *Mol. Microbiol*, **23**, 459-471.

Harris, B.Z., Kaiser, D. and Singer, M. (1998) The guanosine nucleotide (p)ppGpp initiates development and A-factor production in *myxococcus xanthus*. *Genes & Dev*, **12**, 1022-1035.

Hartl, F.U. (1996) Molecular chaperones in cellular protein folding. *Nature*, **381**, 571-579.

Hartzell, P. and Kaiser, D. (1991a) Function of MglA, a 22-kilodalton protein essential for gliding in *Myxococcus xanthus*. *J. Bacteriol*, **173**, 7615-7624.

Hartzell, P. and Kaiser, D. (1991b) Upstream gene of the *mgl* operon controls the level of MglA protein in *Myxococcus xanthus*. *J. Bacteriol*, **173**, 7625-7635.

Hartzell, P.L. (1997) Complementation of sporulation and motility defects in a prokaryote by a eukaryotic GTPase. *Proc. Natl. Acad. Sci. U. S. A*, **94**, 9881-9886.

Hartzell, P.L. and Youderian, P. (1995) Genetics of gliding motility and development in *Myxococcus xanthus*. *Arch. Microbiol*, **164**, 309-323.

Hassett, C., Aicher, L., Sidhu, J.S. and Omiecinski, C.J. (1994) Human microsomal epoxide hydrolase: genetic polymorphism and functional expression in vitro of amino acid variants [published erratum appears in Hum Mol Genet 1994 Jul;3(7):1214]. *Hum. Mol. Genet*, **3**, 421-428.

Hassett, C., Turnblom, S.M., DeAngeles, A. and Omiecinski, C.J. (1989) Rabbit microsomal epoxide hydrolase: isolation and characterization of the xenobiotic metabolizing enzyme cDNA. *Arch. Biochem. Biophys*, **271**, 380-389.

Hecker, M., Schumann, W. and Volker, U. (1996) Heat-shock and general stress response in *Bacillus subtilis*. *Mol. Microbiol*, **19**, 417-428.

Heidelbach, M., Skladny, H. and Schairer, H.U. (1993a) Heat shock and development induce synthesis of a low-molecular-weight stress-responsive protein in the myxobacterium *Stigmatella aurantiaca*. *J. Bacteriol*, **175**, 7479-7482.

Heidelbach, M., Skladny, H. and Schairer, H.U. (1993b) Purification and characterization of SP21, a development-specific protein of the myxobacterium *Stigmatella aurantiaca*. *J. Bacteriol*, **175**, 905-908.

Heikkila, J.J., Krone, P.H. and Ovsenek, N. (1991) Regulation of heat shock gene expression during *Xenopus* development. *Results Probl. Cell Differ*, **17**, 120-137.

Henriques, A.O., Beall, B.W. and Moran, C.P., Jr. (1997) CotM of *Bacillus subtilis*, a member of the alpha-crystallin family of stress proteins, is induced during development and participates in spore outer coat formation. *J. Bacteriol*, **179**, 1887-1897.

Hickey, E., Brandon, S.E., Sadis, S., Smale, G. and Weber, L.A. (1986) Molecular cloning of sequences encoding the human heat-shock proteins and their expression during hyperthermia. *Gene*, **43**, 147-154.

Hodgkin, J. and Kaiser, D. (1979) Genetics of gliding motility and development in *Myxococcus xanthus* (Myxobacterales): two gene control movement. *Mol. Gen. Genet*, **171**, 177-191.

Horwitz, J. (1992) Alpha-crystallin can function as a molecular chaperone. *Proc. Natl. Acad. Sci. U. S. A*, **89**, 10449-10453.

Hull, W.E., Berkessel, A. and Plaga, W. (1998) Structure elucidation and chemical synthesis of stigmolone, a novel type of prokaryotic pheromone. *Proc. Natl. Acad. Sci. U. S. A*, **95**, 11268-11273.

Inouye, M., Inouye, S. and Zusman, D.R. (1979a) Biosynthesis and self-assembly of protein S, a development-specific protein of *Myxococcus xanthus*. *Proc. Natl. Acad. Sci. U. S. A*, **76**, 209-213.

Inouye, M., Inouye, S. and Zusman, D.R. (1979b) Gene expression during development of *Myxococcus xanthus* pattern of protein synthesis. *Dev. Biol*, **68**, 579-591.

Inouye, S. (1990) Cloning and DNA sequence of the gene coding for the major sigma factor from *Myxococcus xanthus*. *J. Bacteriol*, **172**, 80-85.

Inouye, S., Franceschini, T. and Inouye, M. (1983a) Structural similarities between the development-specific protein S from a gram-negative bacterium, *Myxococcus xanthus*, and calmodulin. *Proc. Natl. Acad. Sci. U. S. A*, **80**, 6829-6833.

Inouye, S., Ike, Y. and Inouye, M. (1983b) Tandem repeat of the genes for protein S, a development-specific protein of *Myxococcus xanthus*. *J. Biol. Chem*, **258**, 38-40.

Jakob, U. and Buchner, J. (1994) Assisting spontaneity: the role of Hsp90 and small Hsps as molecular chaperones. *Trends. Biochem. Sci*, **19**, 205-211.

Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) Small heat shock proteins are molecular chaperones. *J. Biol. Chem*, **268**, 1517-1520.

Jakobsen, B.K. and Pelham, H.R. (1988) Constitutive binding of yeast heat shock factor to DNA *in vivo*. *Mol. Cell. Biol*, **8**, 5040-5042.

Jakobsen, B.K. and Pelham, H.R. (1991) A conserved heptapeptide restrains the activity of the yeast heat shock transcription factor. *EMBO. J*, **10**, 369-375.

Jang, Y.J., Park, S.K. and Yoo, H.S. (1996) Isolation of an HSP12-homologous gene of *Schizosaccharomyces pombe* suppressing a temperature-sensitive mutant allele of *cdc4*. *Gene*, **172**, 125-129.

Jobin, M.P., Delmas, F., Garmyn, D., Divies, C. and Guzzo, J. (1997) Molecular characterization of the gene encoding an 18-kilodalton small heat shock protein associated with the membrane of *Leuconostoc oenos*. *Appl. Environ. Microbiol*, **63**, 609-614.

Kadyrzhanova, D.K., Vlachonasios, K.E., Ververidis, P. and Dilley, D.R. (1998) Molecular cloning of a novel heat induced/chilling tolerance related cDNA in tomato fruit by use of mRNA differential display. *Plant. Mol. Biol*, **36**, 885-895.

Kalman, L.V., Cheng, Y.L. and Kaiser, D. (1994) The *Myxococcus xanthus dsg* gene product performs functions of translation initiation factor IF3 in vivo. *J. Bacteriol*, **176**, 1434-1442.

Kato, K., Goto, S., Inaguma, Y., Hasegawa, K., Morishita, R. and Asano, T. (1994) Purification and characterization of a 20-kDa protein that is highly homologous to alpha B crystallin. *J. Biol. Chem*, **269**, 15302-15309.

Keseler, I.M. and Kaiser, D. (1995) An early A-signal-dependent gene in *Myxococcus xanthus* has a sigma 54-like promoter. *J. Bacteriol*, **177**, 4638-4644.

Keseler, I.M. and Kaiser, D. (1997) sigma54, a vital protein for *Myxococcus xanthus. Proc. Natl. Acad. Sci. U. S. A*, **94**, 1979-1984.

Kim, K.K., Kim, R. and Kim, S.H. (1998a) Crystal structure of a small heat-shock protein. *Nature*, **394**, 595-599.

Kim, K.K., Yokota, H., Santoso, S., Lerner, D., Kim, R. and Kim, S.H. (1998b) Purification, crystallization, and preliminary X-ray crystallographic data analysis of small heat shock protein homolog from *Methanococcus jannaschii*, a hyperthermophile. *J. Struct. Biol*, **121**, 76-80.

Kim, R., Kim, K.K., Yokota, H. and Kim, S.H. (1998c) Small heat shock protein of *Methanococcus jannaschii*, a hyperthermophile. *Proc. Natl. Acad. Sci. U. S. A*, **95**, 9129-9133.

Klemenz, R., Andres, A.C., Frohli, E., Schafer, R. and Aoyama, A. (1993) Expression of the murine small heat shock proteins hsp 25 and alpha B crystallin in the absence of stress. *J. Cell. Biol*, **120**, 639-645.

Knack, G., Liu, Z. and Kloppstech, K. (1992) Low molecular mass heat-shock proteins of a light-resistant photoautotrophic cell culture. *Eur. J. Cell. Biol*, **59**, 166-175.

Knauf, U., Bielka, H. and Gaestel, M. (1992) Over-expression of the small heat-shock protein, hsp25, inhibits growth of *Ehrlich ascites* tumor cells. *FEBS Lett*, **309**, 297-302.

Kroos, L. and Kaiser, D. (1987) Expression of many developmentally regulated genes in *Myxococcus* depends on a sequence of cell interactions. *Genes & Dev*, **1**, 840-854.

Kroos, L., Kuspa, A. and Kaiser, D. (1986) A global analysis of developmentally regulated genes in *Myxococcus xanthus*. *Dev. Biol*, **117**, 252-266.

Kuo, Y.P., Thompson, D.K., St. Jean, A., Charlebois, R.L. and Daniels, C.J. (1997) Characterization of two heat shock genes from *Haloferax volcanii* a model system for transcription regulation in the Archaea. *J. Bacteriol*, **179**, 6318-6324.

Kurtz, S., Rossi, J., Petko, L. and Lindquist, S. (1986) An ancient developmental induction: heat-shock proteins induced in sporulation and oogenesis. *Science*, **231**, 1154-1157.

Kuspa, A. and Kaiser, D. (1989) Genes required for developmental signalling in *Myxococcus xanthus*: three *asg* loci. *J. Bacteriol*, **171**, 2762-2772.

Kuspa, A., Plamann, L. and Kaiser, D. (1992a) Identification of heat-stable A-factor from *Myxococcus xanthus*. *J. Bacteriol*, **174**, 3319-3326.

Kuspa, A., Plamann, L. and Kaiser, D. (1992b) A-signalling and the cell density requirement for *Myxococcus xanthus* development. *J. Bacteriol*, **174**, 7360-7369.

Lacourciere, G.M., Vakharia, V.N., Tan, C.P., Morris, D.I., Edwards, G.H., Moos, M. and Armstrong, R.N. (1993) Interaction of hepatic microsomal epoxide hydrolase derived from a recombinant baculovirus expression system with an azarene oxide and an aziridine substrate analogue. *Biochemistry*, **32**, 2610-2616.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Lam, W.Y., Wing Tsui, S.K., Law, P.T., Luk, S.C., Fung, K.P., Lee, C.Y. and Waye, M.M. (1996) Isolation and characterization of a human heart cDNA encoding a new member of the small heat shock protein family-HSPL27. *Biochim. Biophys. Acta*, **1314**, 120-124.

Landry, J., Chretien, P., Laszlo, A. and Lambert, H. (1991) Phosphorylation of HSP27 during development and decay of thermotolerance in Chinese hamster cells. *J. Cell. Physiol*, **147**, 93-101.

LaRossa, R., Kuner, J., Hagen, D., Manoil, C. and Kaiser, D. (1983) Developmental cell interactions of *Myxococcus xanthus*: analysis of mutants. *J. Bacteriol*, **153**, 1394-1404.

Lauzon, L.M., Helm, K.W. and Vierling, E. (1990) A cDNA clone from Pisum sativum encoding a low molecular weight heat shock protein. *Nucleic. Acids. Res*, **18**, 4274.

Lavoie, J.N., Gingras Breton, G., Tanguay, R.M. and Landry, J. (1993) Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. *J. Biol. Chem*, **268**, 3420-3429.

Lee, G.J., Pokala, N. and Vierling, E. (1995) Structure and in vitro molecular chaperone activity of cytosolic small heat shock proteins from pea. *J. Biol. Chem*, **270**, 10432-10438.

Lee, G.J., Roseman, A.M., Saibil, H.R. and Vierling, E. (1997) A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *EMBO. J*, **16**, 659-671.

Lee, K. and Shimkets, L.J. (1994) Cloning and characterization of the *socA* locus which restores development to *Myxococcus xanthus* C-signaling mutants. *J. Bacteriol*, **176**, 2200-2209.

Leroux, M.R., Ma, B.J., Batelier, G., Melki, R. and Candido, E.P. (1997a) Unique structural features of a novel class of small heat shock proteins. *J. Biol. Chem*, **272**, 12847-12853.

Leroux, M.R., Melki, R., Gordon, B., Batelier, G. and Candido, E.P. (1997b) Structure-function studies on small heat shock protein oligomeric assembly and interaction with unfolded polypeptides. *J. Biol. Chem*, **272**, 24646-24656.

Li, S., Lee, B.U. and Shimkets, L.J. (1992) *csgA* expression entrains *Myxococcus xanthus* development. *Genes & Dev*, **6**, 401-410.

Li, S.F. and Shimkets, L.J. (1988) Site-specific integration and expression of a developmental promoter in *Myxococcus xanthus*. *J. Bacteriol*, **170**, 5552-5556.

Liang, P., Amons, R., Macrae, T.H. and Clegg, J.S. (1997) Purification, structure and in vitro molecular-chaperone activity of Artemia p26, a small heat-shock/alpha-crystallin protein. *Eur. J. Biochem*, **243**, 225-232.

Lis, J. and Wu, C. (1992) Transcriptional regulation. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Loomis, W.F. and Wheeler, S.A. (1982) Chromatin-associated heat shock proteins of *Dictyostelium*. *Dev. Biol*, **90**, 412-418.

Lünsdorf, H., Schairer, H.U. and Heidelbach, M. (1995) Localization of the stress protein SP21 in indole-induced spores, fruiting bodies, and heat-shocked cells of *Stigmatella aurantiaca*. *J. Bacteriol*, **177**, 7092-7099.

Mager, W.H. and De Kruijff, A.J. (1995) Stress-induced transcriptional activation. *Microbiol. Rev*, **59**, 506-531.

Magrini, V., Creighton, C. and Youderian, P. (1999) Site-specific recombination of temperate *Myxococcus xanthus* phage Mx8: genetic elements required for integration. *J. Bacteriol*, **181**, 4050-4061.

Magrini, V., Salmi, D., Thomas, D., Herbert, S.K., Hartzell, P.L. and Youderian, P. (1997) Temperate *Myxococcus xanthus* phage Mx8 encodes a DNA adenine methylase, Mox. *J. Bacteriol*, **179**, 4254-4263.

Manoil, C. and Kaiser, D. (1980a) Accumulation of guanosine tetraphosphate and guanosine pentaphosphate in *Myxococcus xanthus* during starvation and myxospore formation. *J. Bacteriol*, **141**, 297-304.

Manoil, C. and Kaiser, D. (1980b) Guanosine pentaphosphate and guanosine tetraphosphate accumulation and induction of *Myxococcus xanthus* fruiting body development. *J. Bacteriol*, **141**, 305-315.

Martin, S., Sodergren, E., Masuda, T. and Kaiser, D. (1978) Systematic isolation of transducing phages for *Myxococcus xanthus*. *Virology*, **88**, 44-53.

Martinez-Argudo, I., Ruiz-Vazquez, R.M. and Murillo, F.J. (1998) The structure of an ECF-δ-dependent, light-inducible promoter from the bacterium *Myxococcus xanthus*. *Mol. Microbiology*, **30**, 883-893.

McCleary, W.R., Esmon, B. and Zusman, D.R. (1991) *Myxococcus xanthus* protein C is a major spore surface protein. *J. Bacteriol*, **173**, 2141-2145.

Meade, H.M., Long, S.R., Ruvkun, G.B., Brown, S.E. and Ausubel, F.M. (1982) Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J. Bacteriol*, **149**, 114-122.

Mehlen, P., Briolay, J., Smith, L., Diaz latoud, C., Fabre, N., Pauli, D. and Arrigo, A.P. (1993) Analysis of the resistance to heat and hydrogen peroxide stresses in COS cells transiently expressing wild type or deletion mutants of the *Drosophila* 27-kDa heat-shock protein. *Eur. J. Biochem*, **215**, 277-284.

Mehlen, P., Hickey, E., Weber, L.A. and Arrigo, A.P. (1997a) Large unphosphorylated aggregates as the active form of hsp27 which controls intracellular reactive oxygen species and glutathione levels and generates a protection against TNFalpha in NIH-3T3-ras cells. *Biochem. Biophys. Res. Commun*, **241**, 187-192.

Mehlen, P., Mehlen, A., Godet, J. and Arrigo, A.P. (1997b) hsp27 as a switch between differentiation and apoptosis in murine embryonic stem cells. *J. Biol. Chem*, **272**, 31657-31665.

Merck, K.B., Groenen, P.J., Voorter, C.E., de Haard Hoekman, W.A., Horwitz, J., Bloemendal, H. and de Jong, W.W. (1993a) Structural and functional similarities of bovine alpha-crystallin and mouse small heat-shock protein. A family of chaperones. *J. Biol. Chem*, **268**, 1046-1052.

Merck, K.B., Horwitz, J., Kersten, M., Overkamp, P., Gaestel, M., Bloemendal, H. and de Jong, W.W. (1993b) Comparison of the homologous carboxy-terminal domain and tail of alpha-crystallin and small heat shock protein. *Mol. Biol. Rep*, **18**, 209-215.

Merrick, M.J. (1993) In a class of its own-the RNA polymerase sigma factor s54 (sN). *Mol. Microbiol*, **10**, 903-909.

Michaud, S., Marin, R. and Tanguay, R.M. (1997) Regulation of heat shock gene induction and expression during *Drosophila* development. *Cell. Mol. Life. Sci*, **53**, 104-113.

Misawa, E., Chan Kwo Chion, C.K., Archer, I.V., Woodland, M.P., Zhou, N.Y., Carter, S.F., Widdowson, D.A. and Leak, D.J. (1998) Characterisation of a catabolic epoxide hydrolase from a *Corynebacterium sp. Eur. J. Biochem*, **253**, 173-183.

Morimoto, R.I. (1993) Cells in stress: transcriptional activation of heat shock genes. *Science*, **259**, 1409-1410.

Morimoto, R.I. (1999) Inducible transcriptional regulation of heat shock genes: the stress signal and the unfolded protein response. In Bukau, B. (ed.) *Molecular chaperones and folding catalysts*. Harwood academic publishers, Amsterdam, pp. 35-52.

Morimoto, R.I., Tissieres, A. and Georgopoulos, C. (eds.) (1994) *The biology of heat shock proteins and molecular chaperones*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Muchowski, P.J. and Clark, J.I. (1998) ATP-enhanced molecular chaperone functions of the small heat shock protein human alphaB crystallin. *Proc. Natl. Acad. Sci. U. S. A*, **95**, 1004-1009.

Murphy, S.P., Gorzowski, J.J., Sarge, K.D. and Phillips, B. (1994) Characterization of constitutive HSF2 DNA-binding activity in mouse embryonal carcinoma cells. *Mol. Cell. Biol*, **14**, 5309-5317.

Nagao, R.T., Czarnecka, E., Gurley, W.B., Schoffl, F. and Key, J.L. (1985) Genes for low-molecular-weight heat shock proteins of soybeans: sequence analysis of a multigene family. *Mol. Cell. Biol*, **5**, 3417-3428.

Nakahigashi, K., Yanagi, H. and Yura, T. (1995) Isolation and sequence analysis of *rpoH* genes encoding sigma 32 homologs from gram negative bacteria: conserved mRNA and protein segments for heat shock regulation. *Nucleic. Acids. Res*, **23**, 4383-4390.

Nakai, A., Kawazoe, Y., Tanabe, M., Nagata, K. and Morimoto, R.I. (1995) The DNA-binding properties of two heat shock factors, HSF1 and HSF3, are induced in the avian erythroblast cell line HD6. *Mol. Cell. Biol*, **15**, 5268-5278.

Nakai, A. and Morimoto, R.I. (1993) Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. *Mol. Cell. Biol*, **13**, 1983-1997.

Nakai, A., Tanabe, M., Kawazoe, Y., Inazawa, J., Morimoto, R.I. and Nagata, K. (1997) HSF4, a new member of the human heat shock factor family which lacks properties of a transcriptional activator. *Mol. Cell. Biol*, **17**, 469-481.

Narberhaus, F., Kaser, R., Nocker, A. and Hennecke, H. (1998) A novel DNA element that controls bacterial heat shock gene expression. *Mol. Microbiol*, **28**, 315-323.

Narberhaus, F., Krummenacher, P., Fischer, H.M. and Hennecke, H. (1997) Three disparately regulated genes for sigma 32-like transcription factors in *Bradyrhizobium japonicum*. *Mol. Microbiol*, **24**, 93-104.

Narberhaus, F., Weiglhofer, W., Fischer, H.M. and Hennecke, H. (1996) The *Bradyrhizobium japonicum rpoH1* gene encoding a sigma 32-like protein is part of a unique heat shock gene cluster together with *groESL1* and three small heat shock genes. *J. Bacteriol*, **178**, 5337-5346.

Nerland, A.H., Mustafa, A.S., Sweetser, D., Godal, T. and Young, R.A. (1988) A protein antigen of Mycobacterium leprae is related to a family of small heat shock proteins. *J. Bacteriol*, **170**, 5919-5921.

Neumann, B., Pospiech, A. and Schairer, H.U. (1992) Size and stability of the genomes of the myxobacteria *Stigmatella aurantiaca* and *Stigmatella erecta*. *J. Bacteriol*, **174**, 6307-6310.

North, A.K., Klose, K.E., Stedman, K.M. and Kustu, S. (1993) Prokaryotic enhancer-binding proteins reflect Eukaryote-like Modularity: the puzzle of nitrogen regulatory protein C. *J. Bacteriol*, **175**, 4267-4273.

O'Connor, K.A. and Zusman, D.R. (1997) Starvation-independent sporulation in *Myxococcus xanthus* involves the pathway for beta-lactamase induction and provides a mechanism for competitive cell survival. *Mol. Microbiol*, **24**, 839-850.

Ogawa, M., Fujitani, S., Mao, X., Inouye, S. and Komano, T. (1996) FruA, a putative transcription factor essential for the development of *Myxococcus xanthus*. *Mol. Microbiol*, **22**, 757-767.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J. and et al. (1992) The alpha/beta hydrolase fold. *Protein. Eng*, **5**, 197-211.

Orlandi, I., Cavadini, P., Popolo, L. and Vai, M. (1996) Cloning, sequencing and regulation of a cDNA encoding a small heat-shock protein from *Schizosaccharomyces pombe*. *Biochim. Biophys. Acta*, **1307**, 129-131.

Orndorff, P., Stellwag, E., Starich, T., Dworkin, M. and Zissler, J. (1983) Genetic and physical characterization of lysogeny by bacteriophage MX8 in *Myxococcus xanthus*. *J. Bacteriol*, **154**, 772-779.

Otani, M., Kozuka, S., Xu, C., Umezawa, C., Sano, K. and Inouye, S. (1998) Protein W, a spore-specific protein in *Myxococcus xanthus*, formation of a large electron-dense particle in a spore. *Mol. Microbiol*, **30**, 57-66.

Parsell, D.A. and Lindquist, S. (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet*, **27**, 437-496.

Pauli, D., Tonka, C.H., Tissieres, A. and Arrigo, A.P. (1990) Tissue-specific expression of the heat shock protein HSP27 during *Drosophila melanogaster* development. *J. Cell. Biol*, **111**, 817-828.

Petko, L. and Lindquist, S. (1986) Hsp26 is not required for growth at high temperatures, nor for thermotolerance, spore development, or germination. *Cell*, **45**, 885-894.

Plaga, W., Stamm, I. and Schairer, H.U. (1998) Intercellular signaling in *Stigmatella aurantiaca*: purification and characterization of stigmolone, a myxobacterial pheromone. *Proc. Natl. Acad. Sci. U. S. A*, **95**, 11263-11267.

Plamann, L., Davis, J.M., Cantwell, B. and Mayor, J. (1994) Evidence that *asgB* encodes a DNA-binding protein essential for growth and development of *Myxococcus xanthus*. *J. Bacteriol*, **176**, 2013-2020.

Plamann, L., Kuspa, A. and Kaiser, D. (1992) Proteins that rescue A-signal-defective mutants of *Myxococcus xanthus*. *J. Bacteriol*, **174**, 3311-3318.

Plamann, L., Li, Y., Cantwell, B. and Mayor, J. (1995) The *Myxococcus xanthus asgA* gene encodes a novel signal transduction protein required for multicellular development. *J. Bacteriol*, **177**, 2014-2020.

Plesofsky Vig, N., Vig, J. and Brambl, R. (1992) Phylogeny of the alpha-crystallin-related heat-shock proteins. *J. Mol. Evol*, **35**, 537-545.

Pries, F., Kingma, J., Pentenga, M., van Pouderoyen, G., Jeronimus Stratingh, C.M., Bruins, A.P. and Janssen, D.B. (1994) Site-directed mutagenesis and oxygen isotope incorporation studies of the nucleophilic aspartate of haloalkane dehalogenase. *Biochemistry*, **33**, 1242-1247.

Punyiczki, M. and Fesus, L. (1998) Heat shock and apoptosis. The two defense systems of the organism may have overlapping molecular elements. *Ann. N. Y. Acad. Sci*, **851**, 67-74.

Qualls, G.T., Stephens, K. and White, D. (1978) Light-stimulated morphogenesis in the fruiting myxobacterium *Stigmatella aurantiaca*. *Science*, **201**, 444-445.

Reichenbach, H. (1984) Myxobactria: A most peculiar group of social prokaryotes. In Rosenberg, E. (ed.) *Myxobacteria, development and cell interactions*. Springer-Verlag New York Inc., New York, pp. 1-51.

Reichenbach, H. (1993) Biologyof the myxobacteria: ecology and taxonomy. In Dworkin, M. and Kaiser, D. (eds.), *Myxobacteria II*. American Society for Microbiology, Washington, D. C., pp. 13-62.

Reichenbach, H. and Dworkin, M. (1992) The order *Myxobacterales*. In Starr, M.P., Trüper, H.G., Balows, A. and Schlegel, H.G. (eds.), *The prokaryotes*. Springer-Verlag, Berlin, pp. 328-355.

Rhodius, V.A. and Busby, S.J. (1998) Positive activation of gene expression. *Curr. Opin. Microbiology*, **1**, 152-159.

Richarme, G. and Caldas, T.D. (1997) Chaperone properties of the bacterial periplasmic substrate-binding proteins. *J. Biol. Chem*, **272**, 15607-15612.

Rink, R., Fennema, M., Smids, M., Dehmel, U. and Janssen, D.B. (1997) Primary structure and catalytic mechanism of the epoxide hydrolase from *Agrobacterium radiobacter* AD1. *J. Biol. Chem*, **272**, 14650-14657.

Roberts, R.C., Toochinda, C., Avedissian, M., Baldini, R.L., Gomes, S.L. and Shapiro, L. (1996) Identification of a *Caulobacter crescentus* operon encoding hrcA, involved in negatively regulating heat-inducible transcription, and the chaperone gene *grpE*. *J. Bacteriol*, **178**, 1829-1841.

Rollet, E., Lavoie, J.N., Landry, J. and Tanguay, R.M. (1992) Expression of *Drosophila*'s 27 kDa heat shock protein into rodent cells confers thermal resistance. *Biochem. Biophys. Res. Commun*, **185**, 116-120.

Romeo, J.M. and Zusman, D.R. (1991) Transcription of the myxobacterial hemagglutinin gene is mediated by a sigma 54-like promoter and a *cis*-acting upstream regulatory region of DNA. *J. Bacteriol*, **173**, 2969-2976.

Rosenbluh, A. and Rosenberg, E. (1989) Autocide AMI rescues development in *dsg* mutants of *Myxococcus xanthus*. *J. Bacteriol*, **171**, 1513-1518.

Ross, W., Aiyar, S.E., Salomon, J. and Gourse, R.L. (1998) *Escherichia coli* promoters with UP elements of different strengths: modular structure of bacterial promoters. *J. Bacteriol*, **180**, 5375-5383.

Ross, W., Gosink, K.K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., Severinov, K. and Gourse, R.L. (1993) A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. *Science*, **262**, 1407-1413.

Rossi, J.M. and Lindquist, S. (1989) The intracellular location of yeast heat-shock protein 26 varies with metabolism. *J. Cell. Biol*, **108**, 425-439.

Roy, S.K. and Nakamoto, H. (1998) Cloning, characterization, and transcriptional analysis of a gene encoding an alpha-crystallin-related, small heat shock protein from the thermophilic cyanobacterium *Synechococcus vulcanus*. *J. Bacteriol*, **180**, 3997-4001.

Ruis, H. and Schuller, C. (1995) Stress signaling in yeast. *Bioessays*, **17**, 959-965.

Salmi, D., Magrini, V., Hartzell, P.L. and Youderian, P. (1998) Genetic determinants of immunity and integration of temperate *Myxococcus xanthus* phage Mx8. *J. Bacteriol*, **180**, 614-621.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (eds.) (1989) *Molecular Cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A*, **74**, 5463-5467.

Santoro, N., Johansson, N. and Thiele, D.J. (1998) Heat shock element architecture is an important determinant in the temperature and transactivation domain requirements for heat shock transcription factor. *Mol. Cell. Biol*, **18**, 6340-6352.

Sarge, K.D., Murphy, S.P. and Morimoto, R.I. (1993) Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress [published errata appear in Mol Cell Biol 1993 May;13(5):3122-3 and 1993 Jun;13(6):3838-9]. *Mol. Cell. Biol*, **13**, 1392-1407.

Sauer, U. and Durre, P. (1993) Sequence and molecular characterization of a DNA region encoding a small heat shock protein of *Clostridium acetobutylicum*. *J. Bacteriol*, **175**, 3394-3400.

Schairer, H.U. (1993) *Stigmatella aurantiaca*, an organism for studying the genetic determination of morphogenesis. In Dworkin, M. and Kaiser, D. (eds.), *Myxobacteria II.* American Society for Microbiology, Washington, D. C., pp. 333-346.

Scharf, K.D., Rose, S., Thierfelder, J. and Nover, L. (1993) Two cDNAs for tomato heat stress transcription factors. *Plant. Physiol*, **102**, 1355-1356.

Schuetz, T.J., Gallo, G.J., Sheldon, L., Tempst, P. and Kingston, R.E. (1991) Isolation of a cDNA for HSF2: evidence for two heat shock factor genes in humans. *Proc. Natl. Acad. Sci. U. S. A*, **88**, 6911-6915.

Schulz, A., Schwab, S., Homuth, G., Versteeg, S. and Schumann, W. (1997) The *htpG* gene of *Bacillus subtilis* belongs to class III heat shock genes and is under negative control. *J. Bacteriol*, **179**, 3103-3109.

Segal, G. and Ron, E.Z. (1996a) Heat shock activation of the *groESL* operon of *Agrobacterium tumefaciens* and the regulatory roles of the inverted repeat. *J. Bacteriol*, **178**, 3634-3640.

Segal, R. and Ron, E.Z. (1996b) Regulation and organization of the groE and dnaK operons in Eubacteria. *FEMS Microbiol. Lett*, **138**, 1-10.

Servant, P. and Mazodier, P. (1995) Characterization of *Streptomyces albus* 18-kilodalton heat shock-responsive protein. *J. Bacteriol*, **177**, 2998-3003.

Shi, W., Kohler, T. and Zusman, D.R. (1993) Chemotaxis plays a role in the social behaviour of *Myxococcus xanthus*. *Mol. Microbiol*, **9**, 601-611.

Shimkets, L. and Woese, C.R. (1992) A phylogenetic analysis of the myxobacteria: basis for their classification. *Proc. Natl. Acad. Sci. U. S. A*, **89**, 9459-9463.

Shimkets, L.J. (1999) Intercellular signaling during fruiting-body development of *Myxococcus xanthus. Annu. Rev. Microbiol*, **53**, 525-549.

Shimkets, L.J. and Asher, S.J. (1988) Use of recombination techniques to examine the structure of the *csg* locus of *Myxococcus xanthus*. *Mol. Gen. Genet*, **211**, 63-71.

Shimkets, L.J. and Rafiee, H. (1990) CsgA, an extracellular protein essential for *Myxococcus xanthus* development. *J. Bacteriol*, **172**, 5299-5306.

Silakowski, B., Ehret, H. and Schairer, H.U. (1998) *fbfB*, a gene encoding a putative galactose oxidase, is involved in *Stigmatella aurantiaca* fruiting body formation. *J. Bacteriol*, **180**, 1241-1247.

Silakowski, B., Pospiech, A., Neumann, B. and Schairer, H.U. (1996) *Stigmatella aurantiaca* fruiting body formation is dependent on the *fbfA* gene encoding a polypeptide homologous to chitin synthases. *J. Bacteriol*, **178**, 6706-6713.

Simon, R., O'Connell, M., Labes, M. and Pühler, A. (1986) Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. *Methods in enzymology*, **118**, 640-658.

Singer, M. and Kaiser, D. (1995) Ectopic production of guanosine penta- and tetraphosphate can initiate early developmental gene expression in *Myxococcus xanthus*. *Genes & Dev*, **9**, 1633-1644.

Sistonen, L., Sarge, K.D., Phillips, B., Abravaya, K. and Morimoto, R.I. (1992) Activation of heat shock factor 2 during hemin-induced differentiation of human erythroleukemia cells. *Mol. Cell. Biol*, **12**, 4104-4111.

Skladny, H., Heidelbach, M. and Schairer, H.U. (1994) Cloning and characterization of the gene encoding the major sigma factor of *Stigmatella aurantiaca. Gene*, **143**, 123-127.

Sorger, P.K. (1990) Yeast heat shock factor contains separable transient and sustained response transcriptional activators. *Cell*, **62**, 793-805.

Sorger, P.K. and Nelson, H.C. (1989) Trimerization of a yeast transcriptional activator via a coiled-coil motif. *Cell*, **59**, 807-813.

Srere, P.A. (1969) Citrate synthase. *Methods in Enzymology*, **XIII**, 3-11.

Stamm, I., Leclerque, A. and Plaga, W. (1999) Purification of cold-shock-like proteins from *stigmatella aurantiaca*-molecular cloning and characterization of the cspA gene. *Arch. Microbiol*, **172**, 175-181.

Stephens, K., Hartzell, P. and Kaiser, D. (1989) Gliding motility in *Myxococcus xanthus*: *mgl* locus, RNA, and predicted protein products. *J. Bacteriol*, **171**, 819-830.

Stephens, K., Hegeman, G.D. and White, D. (1982) Pheromone produced by the myxobacterium *Stigmatella aurantiaca*. *J. Bacteriol*, **149**, 739-747.

Surewicz, W.K. and Olesen, P.R. (1995) On the thermal stability of alpha-crystallin: a new insight from infrared spectroscopy. *Biochemistry*, **34**, 9655-9660.

Takahashi, T. and Komeda, Y. (1989) Characterization of two genes encoding small heat-shock proteins in *Arabidopsis thaliana*. *Mol. Gen. Genet*, **219**, 365-372.

Tanabe, M., Nakai, A., Kawazoe, Y. and Nagata, K. (1997) Different thresholds in the responses of two heat shock transcription factors, HSF1 and HSF3. *J. Biol. Chem*, **272**, 15389-15395.

Teintze, M., Thomas, R., Furuichi, T., Inouye, M. and Inouye, S. (1985) Two homologous genes coding for spore-specific proteins are expressed at different times during development of *Myxococcus xanthus. J. Bacteriol*, **163**, 121-125.

Toal, D.R., Clifton, S.W., Roe, B.A. and Downard, J. (1995) The esg locus of *Myxococcus xanthus* encodes the E1 alpha and E1 beta subunits of a branched-chain keto acid dehydrogenase. *Mol. Microbiol*, **16**, 177-189.

Tojo, N., Sanmiya, K., Sugawara, H., Inouye, S. and Komano, T. (1996) Integration of bacteriophage Mx8 into the *Myxococcus xanthus* chromosome causes a structural alteration at the C-terminal region of the IntP protein. *J. Bacteriol*, **178**, 4004-4011.

Ueki, T. and Inouye, S. (1998) A new sigma factor, SigD, essential for stationary phase is also required for multicellular differentiation in *Myxococcus xanthus. Genes. Cells*, **3**, 371-385.

van den, I.P.R., Overkamp, P., Knauf, U., Gaestel, M. and de Jong, W.W. (1994) Alpha A-crystallin confers cellular thermoresistance. *FEBS Lett*, **355**, 54-56.

Veinger, L., Diamant, S., Buchner, J. and Goloubinoff, P. (1998) The small heat-shock protein IbpB from Escherichia coli stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J. Biol. Chem*, **273**, 11032-11037.

Ward, M.J., Lew, H., Treuner-Lange, A. and Zusman, D.R. (1998) Regulation of motility behavior in *Myxococcus xanthus* may require an extracytoplasmic-function sigma factor. *J. Bacteriol*, **180**, 5668-5675.

Ward, M.J. and Zusman, D.R. (1997) Regulation of directed motility in *Myxococcus xanthus*. *Mol. Microbiol*, **24**, 885-893.

Waters, E.R. (1995) The molecular evolution of the small heat-shock proteins in plants. *Genetics*, **141**, 785-795.

Waters, E.R. and E., V. (1999) The diversification of plant cytosolic small heat shock proteins preceded the divergence of mosses. *Mol. Biol. Evol*, **16**, 127-139.

Welsh, M.J. and Gaestel, M. (1998) Small heat-shock protein family: function in health and disease. *Ann. N. Y. Acad. Sci*, **851**, 28-35.

Wösten, M.M.S.M. (1998) Eubacteria sigma factors. *FEMS Microbiology Reviews*, **22**, 127-150.

Wotton, D., Freeman, K. and Shore, D. (1996) Multimerization of Hsp42p, a novel heat shock protein of *Saccharomyces cerevisiae*, is dependent on a conserved carboxyl-terminal sequence. *J. Biol. Chem*, **271**, 2717-2723.

Wu, C. (1995) Heat shock transcription factors: structure and regulation. *Ann. Rev. Cell. Dev. Biol*, **11**, 441-469.

Xiao, H. and Lis, J.T. (1988) Germline transformation used to define key features of heat-shock response elements. *Science*, **239**, 1139-1142.

Yeh, C.H., Chang, P.F., Yeh, K.W., Lin, W.C., Chen, Y.M. and Lin, C.Y. (1997) Expression of a gene encoding a 16.9-kDa heat-shock protein, Oshsp16.9, in *Escherichia coli* enhances thermotolerance. *Proc. Natl. Acad. Sci. U. S. A*, **94**, 10967-10972.

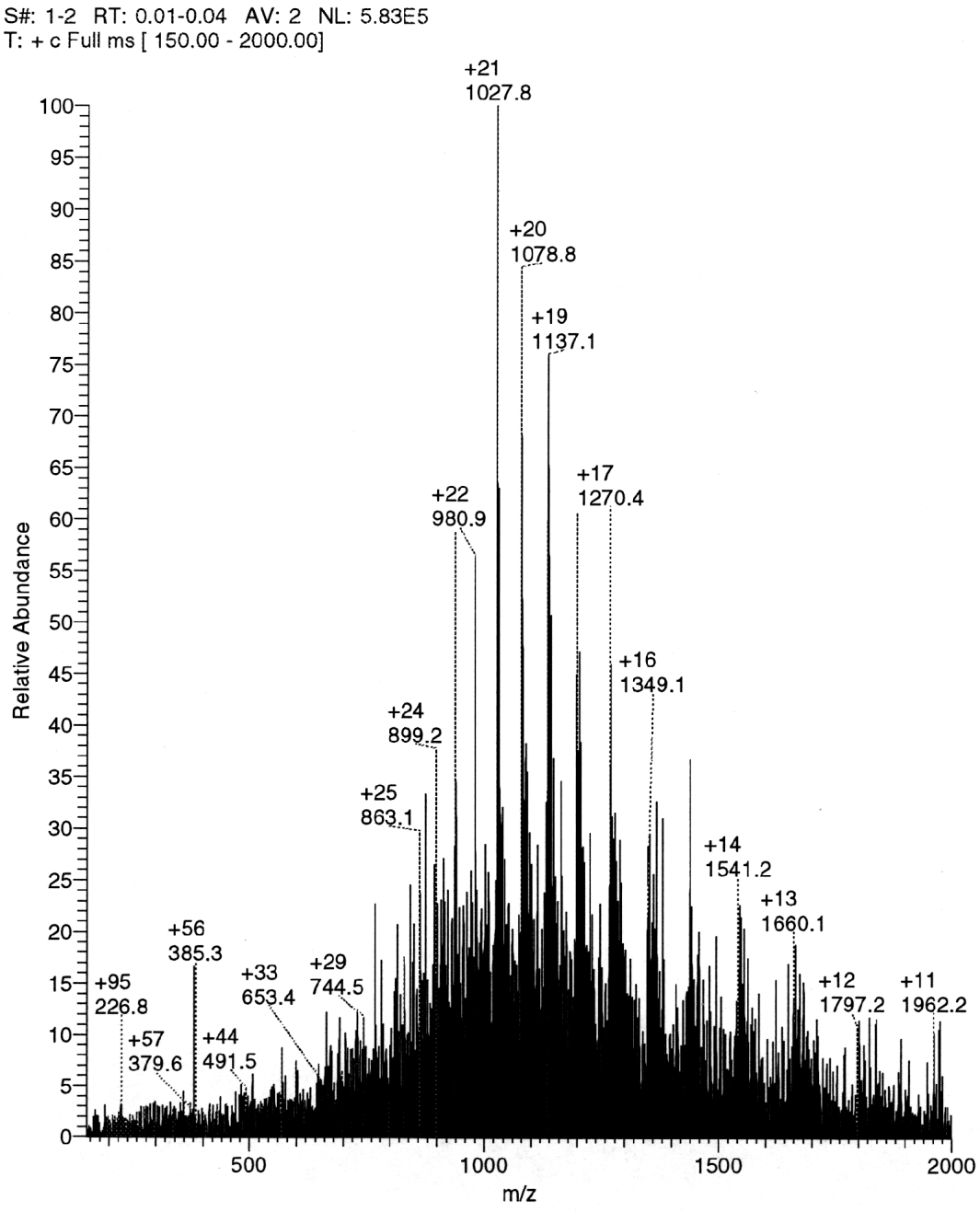
Youderian, P. (1998) Bacterial motility: Secretory secrets of gliding bacteria. *Current Biology*, R408-411.

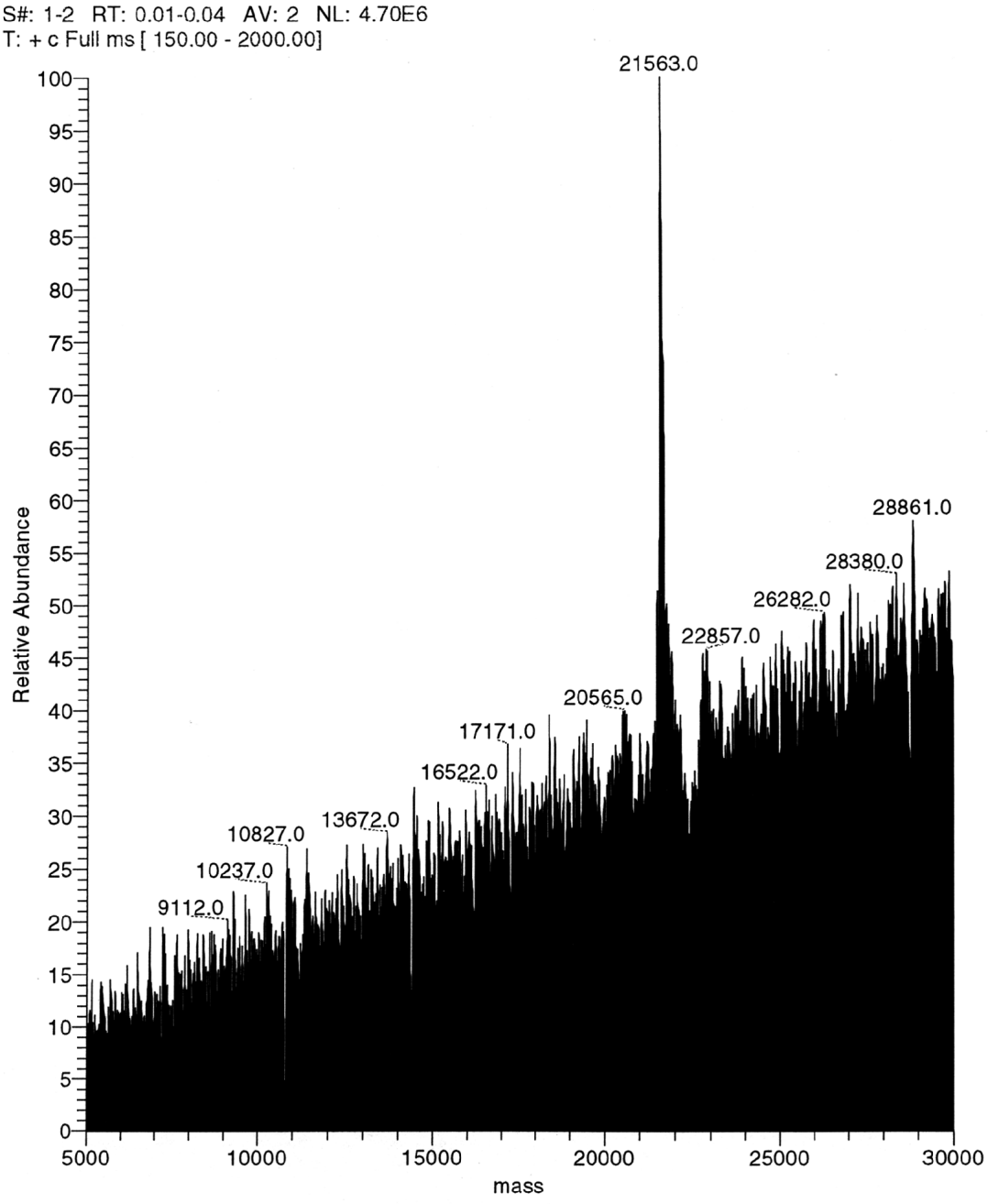
Yura, T., Nagai, H. and Mori, H. (1993) Regulation of the heat-shock response in bacteria. *Annu. Rev. Microbiol*, **47**, 321-350.

Zhang, W., Inouye, M. and Inouye, S. (1996) Reciprocal regulation of the differentiation of *Myxococcus xanthus* by Pkn5 and Pkn6, eukaryotic-like Ser/Thr protein kinases. *Mol. Microbiol*, **20**, 435-447.

VII. Appendices

Appendix 1. Mass spectrometry of $HspA_{His}$ monomer




S#: 1-2 RT: 0.01-0.04 AV: 2 NL: 4.70E6
T: + c Full ms [150.00 - 2000.00]
Relative Abundance
mass
21563.0
28861.0
28380.0
26282.0
22857.0
20565.0
17171.0
16522.0
13672.0
10827.0
10237.0
9112.0
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
5000
10000
15000
20000
25000
30000

Appendix 2. Abbrevations

Amp^r	ampicillin resistance
ATP	adenosine 5'-triphosphate
attP	attachment site of phage
attB	attachment site of bacterium
bp	base pair
dATP	deoxyadenosine 5'-phosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dNTPαS	2'-deoxyribonucleoside 5'-O-(1-thiotriphosphates)
Fig.	figure
E. coli	*Escherichia coli*
ß-gal.	ß-galactosidase
HPLC	high performance liquid chromatography
hr	hour
HSP	heat shock protein
IgG	immunoglobulin G
Km^r	kanamycin resistance
kbp	kilobasepair
kDa	kilodalton
log	logarithmic
M	molar
mg	miligram
min	minute
mM	milimolar
mRNA	messenger ribonucleic acid
nts	nucleotides
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
P*hspA*	the *hspA* promoter

RBS	ribosome binding site
rel.	relative
RNA	ribonucleic acid
RNase	ribonuclease
Str^r	streptomycin resistance.
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar