

I. *Stigmatella aurantiaca*, a Prokaryotic Organism for Studying Intercellular Signalling and Morphogenesis

The organisms

Stigmatella aurantiaca belongs to the myxobacteria that are Gram-negative soil bacteria. Myxobacteria show both, features of unicellular and multicellular organisms. As the eukaryotic organism *Dictyostelium discoideum* they are thought to lie on the boundary between unicellular and multicellular organisms. Myxobacteria grow and divide as separate cells. But they may be regarded as a multicellular organisms whose cells feed in swarms and which under conditions of starvation, assemble to well defined regular three dimensional structures called fruiting bodies which enclose about 10^5 dormant cells, the myxospores. The shape of the fruiting bodies is species specific and is genetically determined. The fruiting body of *S. aurantiaca* consists of a stalk bearing several sporangioles on branches at its top.

Myxobacteria secrete hydrolytic enzymes together with slime with which they degrade particulate organic matter of the soil. It has been shown that the growth rate increases with cell density if myxobacteria were grown on a macromolecular substrate as sole nutrient, such as casein. This suggests that cells feed co-operatively and the association in a swarm allows them to feed more efficiently. The advantage of cooperative feeding may have driven the evolution of fruiting body formation. When nutrients are again available after a period of starvation, myxospores germinate and form vegetative cells. The multicellular nature of the fruiting body ensures that a swarm of cells is formed for a new growth cycle.

Myxobacteria move by gliding on solid surfaces. This

facilitates the stabilisation of a swarm and of fruiting body formation. Gliding permits tight cell-cell contact and efficient signal exchange between the cells by diffusible molecules. Both features are a prerequisite for the transmission of positional information of the single cell necessary for the coordination of the metabolism and movement of the cell in the course of fruiting body formation. One of the developmental signals – Stigmolone – that is involved in early cell aggregation has been recently isolated and characterised by Wulf Plaga et al.

Apart from their ability to form fruiting bodies, myxobacteria form a broad range of secondary metabolites. All these unique features are reflected in the size of the genome and its organisation. The size of the myxobacterial genome has been shown to be about 9.5 Mbp.

A gene cluster involved in *S. aurantiaca* fruiting body formation

Susanne Müller, Barbara Silakowski and Diana Hofmann

To investigate the genes involved in *S. aurantiaca* fruiting body formation and the co-ordination of their expression, Tn5 transposon insertion mutagenesis was performed. Three different mutant types impaired in fruiting body formation were detected by screening the insertional mutants. They include mutants that form neither fruiting bodies nor aggregates, mutants that aggregate to unstructured clumps, and mutants that undergo only part of the differentiation process. One of the mutants (AP182) that formed clumps during starvation was analysed further. Mixing of the

cells of this strain with those of a mutant (AP191), which was unable to form aggregates prior to starvation, lead to a partial phenotypic complementation. Instead of clumps, a mushroom-like structure, similar to a champignon was obtained. Sequencing of the mutant gene of strain AP182 and of the adjacent genomic segments resulted in four open reading frames that were involved in fruiting body formation. One of the genes, *fbfB*, and the other three genes, *fbfA*, *fbfC*, and *fbfD* are arranged in a divergent orientation. FbfB shows significant homology to the secreted copper enzyme galactose oxidase from the fungus *Dactylium dendroides*. *fbfA* encodes a polypeptide that is homologous to chitin synthases. The start codon of *fbfC* overlaps with the stop codon of *fbfA*. FbfC has no significant homology to any of the known proteins. FbfD has similarities to the human phosphoprotein synapsin I. Inactivation of either of these genes by insertion of the *neo* gene cassette resulted in mutants that formed only unstructured clumps during starvation. This indicates the gene products of the *fbf*-genes to be involved in fruiting body formation. No additional open reading frame involved in fruiting was detected downstream of *fbfD*. Mixing of cells from either the *fbfA* or the *fbfB* mutant with those of the non-aggregating strain AP191 before starvation resulted in mushroom-like fruiting bodies with the form of a morel or a champignon, respectively. These forms are observed during wild-type fruiting body formation 12 or 15 hours after the beginning of starvation.

The partial phenotypic complementation suggests that factors involved in fruiting and which are lacking in one mutant may be obtained from the other. A reason for the incomplete phenotypic complementation may be that not all substances involved in fruiting (e.g., intracellular macromolecules) which are lacking in

one of the strains can be supplemented by the other mutant and vice versa.

For the analysis of the time dependence of the expression of the genes *fbfA*, *fbfB*, *fbfC*, and *fbfD* during fruiting body formation or indole induced sporulation merodiploid strains were constructed. They harboured the wild type genes and in addition a 3' truncated *fbf*-gene with 5' regions of variable length. The truncated genes were fused to the promoterless hybrid indicator gene $\Delta trp-lacZ$ and the *neo* gene for selection. β -Galactosidase activity increased 8 or 14 hours after the beginning of starvation in the merodiploid strains but not during indole induced sporulation. This unequivocally proves the four *fbf*-genes to be involved in the morphogenic process of fruiting. RT-PCR analyses of *fbf*-gene transcription revealed these genes to be induced during starvation. Low levels of *fbf*-gene transcript are found in vegetative cell and in the case of *fbfC* or *fbfD* during indole induced sporulation. Analysis of the protein patterns of the wild-type and the mutant strains by 2D electrophoresis is in progress.

Alternative sigma factors

Barbara Silakowski, Susanne Müller and Chi-Hyuk Chang

The genes of two alternative sigma factors, *sigB* and *sigC* have been cloned. These sigma factors harbour two domains that were shown for σ^{32} of *E. coli* to be necessary for DnaK binding and thus for its proteolytic clearance. Merodiploid strains containing the wild type gene and the corresponding 3' truncated gene fused to an indicator gene were analysed for the expression of the sigma factor genes during development or heat shock. *sigB* was shown to be expressed early during indole-induced sporulation and fruiting body formation as well during heat shock. These

results agree with those of the RT-PCR analysis of *sigB* transcription. Inactivation of either *sigB* or *sigC* by insertional mutagenesis did not impair fruiting body formation, indole-induced sporulation or the heat shock response. No changes in either the spores' ultrastructure (H. Lünsdorf, GBF, Braunschweig) or in spore germination have been detected.

A gene cluster of *S. aurantiaca* DW4/3-1 for Myxothiazol biosynthesis

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Sequence analyses downstream of the developmental *fbfB* gene resulted in the detection of the *mta* (myxothiazol) gene cluster. The first ORF, *mtaA*, (formerly designated *hesA*), encodes a phosphopantetheinyl transferase. P-pant transferases activate polyketide synthases (PKS) by the transfer of the P-pant moiety from coenzyme A to a conserved serine residue of the PKS. Downstream of *mtaA* a second ORF, *mtaB*, (formerly *pksA*) was found, of which 7 kbp were sequenced. It encodes a PKS. Inactivation of *mtaA* by insertional mutagenesis or deletion of part of the gene or insertional inactivation of *mtaB* gene impairs myxothiazol synthesis. In addition mutants defective in *mtaA* fail to form a sofar unknown metabolite. This suggests MtaA to be able to activate at least two different polyketide synthases.

For studying the expression of the *mtaB* gene a merodiploid strain BS64 was constructed that harboured a functional wildtype and a 3' truncated *mtaB* gene to which an indicator gene was fused. Measurement of indicator gene expression showed, *mtaB* to be expressed under all conditions tested, such as vegetative growth, fruiting, indole induced sporulation and heat shock. The project was stopped at this stage at the

ZMBH. Further investigations of the gene cluster are performed at the GBF in Braunschweig.

CspA, a prominent cold-shock(-like) protein

I. Stamm and W. Plaga

Several related proteins of about 7 kDa constitute a prominent fraction of the *S. aurantiaca* cell extract. One of the genes encoding such a protein was cloned and named *cspA*. The delineated protein sequence of 68 amino acid residues displays a high sequence identity with bacterial cold-shock(-like) proteins. A RNA chaperon-function was proposed for these proteins in *E. coli*. Using a *cspA::(trp-lacZ)* fusion gene that was introduced into *Stigmatella* by electroporation the transcription was analyzed during development and at lowered temperature. These experiments indicated *cspA* to be constitutively transcribed at a high level. The *cspA* promoter was used to express the gene for the green fluorescent protein (GFP). GFP fluorescence was found to be detectable in whole fruiting bodies as well as in single cells. The GFP-labelled cells are easily distinguished from wild type cells using a fluorescence-activated cell sorter (FACS). Fruiting body formation is not impaired in *gfp* expressing wild type strains. Thus the *gfp* gene under the control of the *cspA* promoter seems to be suitable to label *Stigmatella* strains. With this kind of labelling it should be possible to analyze the fate of mutant strain cells in phenotypic complementation experiments during fruiting body formation; in such experiments fruiting bodies are formed by the combined action of two mutant strains which are unable to develop properly on their own.

Fluorescence based analysis of gene expression: Identification of pheromone target genes

The pheromone stigmolone (2,5,8-trimethyl-8-hydroxy-nonan-4-one) is instrumental in early steps of fruiting body formation. To identify stigmolone-responsive genes a promoter trap vector (pTRAP1) was constructed which allows the creation of random promoter fusions to *gfp* in *S. aurantiaca*. With the aid of a flow cytometer a selection strategy exploiting the differential fluorescence induction (DFI) of these promoter fusions by stigmolone is feasible. First analyses of the random insertion mutants by flow cytometry have shown, that about 2.5% of the mutants express the *gfp* during vegetative growth. Screening for genes affected by the addition of stigmolone is in progress.

HspA (SP21): Transcriptional regulation and biological function

Hui Shen

HspA (formerly SP21) of *S. aurantiaca* is synthesised during development or under stress such as heat shock, oxygen limitation or indole treatment. Sequence alignment revealed HspA to belong to the α -crystallin family of the low molecular weight heat shock proteins. HspA is located mainly at the cell periphery in heat shocked cells and in fruiting body derived myxospores and either at the cell periphery or within the cytoplasm of indole treated cells as shown by immunoelectron microscopy (H. Lünsdorf, GBF, Braunschweig).

Two transcripts of the *hspA* gene were detected after heat shock and only one after indole treatment. A unique transcription initiation site of the monocistronic *hspA* gene was detected by primer extension either after heat shock or indole treatment. Analysis

of the promoter proved various upstream regions to be required for maximum expression of *hspA* under stress conditions. For maximum *hspA* transcription 225 bp and 587 bp upstream of the ATG start codon are required in the case of heat shock or indole treatment, respectively.

To delimit the regulatory elements involved in *hspA* transcription that depends on heat shock, deletion / insertion mutagenesis as well as gel shift assays were performed. Three regulatory promoter regions involved in heat shock response were defined. The first region spans from bp -56 to bp -85 upstream of the *hspA* ORF and harbours the RNA polymerase binding sites. Deletion of this region completely blocks *hspA* transcription. The second domain ranges from bp -141 to bp -223 upstream of the *hspA* ORF and carries putative regulator-binding sites. Heat shock and phosphorylation enhance binding of the regulator(s) to the *hspA* promoter. Deletion of this region reduces *hspA* transcription by more than half. Deleting a sequence ranging from bp -86 to bp -140 upstream of the *hspA* ORF abolishes *hspA* transcription suggesting a *cis*-acting element to exist just upstream of the -35, -10 regions of the *hspA* promoter. These results suggest the transcription of *hspA* to be mainly positively regulated under heat shock conditions.

A His-tagged fusion protein of HspA (HspA_{His}) was produced in *E. coli*. This polypeptide tends to assemble into a large complex that consists of 26 subunits with a molecular mass of 560 kDa as judged by size exclusion chromatography. This oligomer of HspA_{His} interacts with unfolded cytrate synthetase (CS) and prevents the enzyme's precipitation. The unfolded B-chain of insulin is not protected from precipitation. A stable complex is formed between HspA_{His} and unfolded CS because the unfolded enzyme does not dissociate from the complex. Though thermotoler-

ance and differentiation of *S. aurantiaca* cells are not affected in the absence of this protein, one may suggest HspA to play a protective role *in vivo*.

II. Molecular Biology of the Infection Process by the Entomopathogenic Fungus *Beauveria bassiana*

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The organism

The filamentous fungus *Beauveria bassiana* (Balsamo) Vuillemin belongs to a class of insect pathogenic hyphomycetes (fungi imperfecti). The different *Beauveria* strains are highly adapted to particular host insects. A broad range of *B. bassiana* species is found world wide under very different climatic conditions. The different *Beauveria* strains cover a rather wide spectrum of insect hosts that are of medical or agricultural significance. Hosts of medical importance include vectors for agents of tropical infectious diseases such as the tsetse fly *Glossina morsitans morsitans*, the sand fly *Phlebotomus* that transmits *Leishmania*, and bugs of the genera *Triatoma* and *Rhodnius*, the vectors of Chagas' disease. Hosts of agricultural significance include the Colorado potato beetle *Leptinotarsa decemlineata*, the codling moth *Carpocapsa pomonella* and several genera of termites.

In the absence of the specific insect host, the facultative insect pathogen passes through an asexual vegetative life cycle that includes germination, filamentous growth, and the formation of sympoduloconidia from poorly differentiated conidiophores. In the presence of its host insect, *Beauveria* switches to the pathogenic

life cycle. The conidiospores germinate on the surface of the cuticle; the newly generated hyphae penetrate the insect's integument and liberate single cells, so-called blastospores, or hyphal bodies, when reaching the hemocoel. The hemolymph distributes the blastospores through the whole body cavity, that thus form points of origin of new hyphae that invade all host tissues. When the nutrients of the carcass are used up, a thick layer of aerial hyphae is formed on the surface of the insect's cadaver, of which conidiospores are released.

During the infectious process fungal structures are subject to several defense response mechanisms of the host-insect. These include humoral as well as hemocytic encapsulation reactions, hemolymph clotting reactions with the aim to hinder blastospore propagation, and the action of defensin-like small antifungal peptides.

To overcome the host's defense mechanisms and physical barriers it is suggested the parasite to have two classes of virulence factors. One class comprises the more general virulence factors that are independent of the host insect. They include hydrophobin like proteins that mediate spore adhesion to cuticular structures, lytic enzymes such as proteases or chitinases for cuticle penetration and hemocoel invasion and diffusible cyclopeptide- or depsipeptide-toxins that suppress the defense reactions of the host. The second class comprises factors that are needed for the switch from saprophytic growth to the growth on a specific host-insect. These factors include a specific receptor- and signal-transduction-system that allows the fungus to differentiate between its correct host and other insects.

Characterisation of genes involved in *B. bassiana* virulence

For the identification of virulence factors of a *B. bassiana* strain that is highly adapted to the Colorado potato beetle, two complementary strategies are employed. Firstly, in a REMI (“restriction enzyme mediated integration”) transformation approach mutants are generated. Mutants that result from a monolocal recombination event are tested for their ability to grow on their host-insect. In the case of a significant reduction of their virulence the disrupted genomic region will be cloned and sequenced. Secondly, a mRNA differential display analysis is performed to identify fungal genes that are transcriptionally up regulated by the interaction with the host using cultures grown in the absence or presence of host structures. These genes are cloned from a wild type genomic DNA library. The significance of the thus found potential virulence factor genes for pathogenesis are tested by site-specific inactivation of the single cloned genes or of groups of functionally related genes and the determination of the pathogenicity phenotype of the corresponding null mutants.

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THESES

Dissertation

Shen, Hui (1999). Transcriptional regulation of *hspA* gene in *Stigmatella aurantiaca* and function analysis of HspA protein. Universität Heidelberg.

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