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**CsgA, ein putatives Signalmolekül der
Fruchtkörperbildung in dem Myxobakterium
Stigmatella aurantiaca:
Charakterisierung des *csgA*-Gens und des Einflusses
seiner Inaktivierung auf die Entwicklung**

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**CSGA, A PUTATIVE SIGNAL MOLECULE OF THE
MYXOBACTERIUM *Stigmatella aurantiaca*
INVOLVED IN FRUITING:
Characterization of the *csgA* gene and influence of
csgA inactivation on development**

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Contents

I. Introduction	1
1.1. Myxobacteria.....	3
1.2. Gliding motility	4
1.2.1. Gliding motility of myxobacteria	4
1.2.1.1. A system.....	5
1.2.1.2. S system.....	5
1.2.1.3. The <i>mgl</i> locus.....	6
1.2.1.4. The <i>frz</i> locus	6
1.2.1.5. Rippling.....	7
1.3. Fruiting body formation.....	7
1.3.1. Pheromone activity in <i>S. aurantiaca</i>	8
1.3.2. Artificially induced sporulation.....	9
1.3.3. Genes involved in <i>S. aurantiaca</i> fruiting body formation	9
1.4. Intercellular signalling and communication in bacteria	11
1.4.1. Intercellular signalling in Gram-negative bacteria	12
1.4.2. Intercellular signalling in Gram-positive bacteria	12
1.4.3. Intercellular signalling in <i>M. xanthus</i>	13
1.4.3.1. A signalling.....	13
1.4.3.2. B signalling.....	13
1.4.3.3. C signalling.....	14
1.4.3.4. D signalling.....	16
1.4.3.5. E signalling.....	17
1.4.4. Intercellular signalling in <i>S. aurantiaca</i>	17
1.5. The aims of this work	17
II. Results.....	19
2.1. Molecular cloning and sequence analysis of the <i>csgA</i> locus from <i>S. aurantiaca</i>	20
2.1.1. Cloning of the <i>csgA</i> locus from <i>S. aurantiaca</i>	21
2.1.1.1. Preparation and screening of a <i>S. aurantiaca</i> genomic library.....	22
2.1.1.2. Subcloning of the 3 kbp XhoI fragment harbouring the <i>S. aurantiaca</i> <i>csgA</i> gene.....	23
2.1.1.3. Subcloning of the XhoI <i>csgA</i> fragment from λ 11 into the plasmid pACYC177.....	24
2.1.2. Sequence analysis of the <i>csgA</i> locus from <i>S. aurantiaca</i>	24
2.1.2.1. Analysis of the upstream and downstream sequences of <i>csgA</i>	29
2.2. Investigation of the physiological function of CsgA in vivo;.....	30
Disruption of the <i>csgA</i> gene in <i>S. aurantiaca</i>	30
2.2.1. Construction of plasmid pAM8	30
2.2.2. Construction of the <i>csgA</i> insertion mutant AM8	31
2.2.3. Developmental phenotype of the <i>csgA</i> insertion mutant strain	34
2.2.4. The ability of AM8 myxospores to germinate	37
2.2.5. Ability of the wild type to restore developmental phenotype of mutant AM8	37
2.2.6. Interaction between WP120 and AM8 mutant cells.....	38
2.3. Transcription of <i>csgA</i> in <i>S. aurantiaca</i>	40
2.3.1. Determination of the <i>csgA</i> expression in the merodiploid mutant AM14....	40
2.3.1.1. Construction of plasmid pAM14	40
2.3.1.2. Constructions of the merodiploid mutant strain AM14	41
2.3.1.3. Determination of the β -galactosidase activity in AM14	42
2.3.1.4. Detection of the β -galactosidase activity <i>in situ</i>	44

Contents

2.3.1.5. Developmental phenotype of AM14.....	45
2.3.2. Detection of <i>csgA</i> expression by RT-PCR	46
2.4. Production of CsgA in <i>S. aurantiaca</i>	46
2.4.1. Heterologous expression of the fragment encoding antigenic determinants of CsgA	47
2.4.1.1. Cloning the DNA fragment encoding the CsgA antigens into the pQE42 expression vector	47
2.4.1.2. Purification of the recombinant protein, 6xHis-DHFR-CsgA, under denaturing conditions	49
2.4.1.3. Production of CsgA in <i>S. aurantiaca</i>	50
2.4.1.5. Production of anti-peptide antibodies	52
III. Discussion.....	57
3.1. <i>S. aurantiaca csgA</i> gene locus	58
3.2. CsgA protein and similarity with members of the SRD family.....	61
3.3. Physiological function of CsgA	62
3.4. Expression of the <i>csgA</i> gene in <i>S. aurantiaca</i>	67
3.5. Immunological identification of CsgA in <i>S. aurantiaca</i>	69
3.6. Perspectives.....	70
IV. Materials and Methods	72
4.1. Materials	73
4.1.1. Specified chemicals, consumables and equipments	73
4.1.1.1. Chemicals	73
4.1.1.2. Consumables.....	74
4.1.1.3. Equipment.....	74
4.1.2. Protein	74
4.1.2.1. Antibodies.....	74
4.1.2.2. Protein weight standards	74
4.1.3. Reagent kits for methods in molecular biology and enzymes.....	75
4.1.3.1. Reagent kits for methods in molecular biology	75
4.1.3.2. Enzymes	75
4.1.4. Nucleic acids	75
4.1.4.1. Plasmid	75
4.1.4.2. Primers	75
4.1.4.3. Oligonucleotides	76
4.1.4.4. DNA and RNA molecular weight markers	76
4.1.5. Bacterial strains	76
4.1.5.1. <i>Escherichia coli</i>	76
4.1.5.2. <i>S. aurantiaca</i> strain	77
4.1.6. Media and stocks solutions	77
4.1.6.1. Media.....	77
4.1.6.2. Buffers and stock solutions	77
4.2. Methods	78
4.2.1. Microbiologic techniques.....	78
4.2.1.1. Growth of <i>E. coli</i>	78
4.2.1.2. Growth of <i>S. aurantiaca</i>	78
4.2.1.3. Indol induced sporulation of <i>S. aurantiaca</i> (Gerth and Reichenbach, 1994).....	78
4.2.1.4. Heat shock of <i>S. aurantiaca</i>	78
4.2.1.5. <i>S. aurantiaca</i> fruiting body formation assay	79

Contents

4.2.1.6. Germination of <i>S. aurantiaca</i> spores	79
4.2.1.7. Preservation of <i>E. coli</i> cultures	79
4.2.1.8. Preservation of <i>S. aurantiaca</i> cultures	79
4.2.1.9. Electroporation.....	79
4.2.1.9.1. Electroporation of <i>E. coli</i>	80
4.2.1.9.2. Electroporation of <i>S. aurantiaca</i> (Stamm et al., 1999)	80
4.2.1.10. Blue white colony screening selection of <i>E. coli</i>	80
4.2.1.11. Preparation of bacteriophage host <i>E. coli</i> cells.....	81
4.2.1.12. Infection of <i>E. coli</i> with the bacteriophages.....	81
4.2.1.13. Titering of the phage library.....	81
4.2.1.14. Amplification of the <i>S. aurantiaca</i> genomic phage library.....	82
4.2.1.15. Plaque lifts	82
4.2.1.16. Purification of bacteriophage clones.....	82
4.2.2. Isolation and manipulation of DNA	83
4.2.2.1. Isolation of plasmid DNA from <i>E. coli</i>	83
4.2.2.1.1. Isolation of plasmid DNA from <i>E. coli</i> cells with alkaline lyses	83
4.2.2.1.2. Isolation of plasmid DNA from <i>E. coli</i> cells with anion exchange columns.....	83
4.2.2.2. Isolation of chromosomal DNA from <i>S. aurantiaca</i> (Neumann et al., 1992).....	83
4.2.2.3. Isolation of lambda DNA	84
4.2.2.4. Phenol/chloroform extraction of DNA (Sambrook et al., 1989)	84
4.2.2.5. Alcohol precipitation of DNA	84
4.2.2.6. Quantitation of DNA.....	84
4.2.2.7. DNA restriction.....	84
4.2.2.8. Partial digestion of DNA.....	85
4.2.2.9. DNA ligation	85
4.2.2.10. Construction of the <i>S. aurantiaca</i> phage library.....	85
4.2.2.11. Filling of 5` overhanging ends of DNA	85
4.2.2.12. Removing of 3` overhanging ends of DNA	86
4.2.2.13. Dephosphorylation of DNA fragments	86
4.2.2.14. Amplification of DNA fragments via PCR (polymerase chain reaction)	86
4.2.2.15. Purification of PCR products.....	86
4.2.2.16. DNA sequencing.....	87
4.2.3. Electrophoresis of DNA.....	87
4.2.3.1. Agarose gel electrophoresis of DNA	87
4.2.3.2. Recovery of DNA fragments from low-melting agarose gel	87
4.2.4. DNA hybridisation.....	87
4.2.4.1. Dot blot analysis	87
4.2.4.2. Southern blot analysis (Southern, 1975)	88
4.2.4.3. Hybridisation and detection with biotin-labelled probes	88
4.2.4.4. Nonradioactive labelling of DNA probes.....	89
4.2.5. Isolation and manipulation of RNA.....	89
4.2.5.1. Isolation of RNA from <i>S. aurantiaca</i> cells.....	89
4.2.5.2. RNA electrophoresis	90
4.2.5.3. RT-PCR	90
4.2.6. Protein purification and analysis	90
4.2.6.1. Isolation of total protein extract from <i>S. aurantiaca</i>	90

Contents

4.2.6.2. Determination of protein concentration (Bradford, 1976)	91
4.2.6.3. SDS-Polyacrilamide gel electrophoresis (Laemmli, 1970).....	91
4.2.6.4. Coomassie blue staining of gels	91
4.2.6.5. Immunoblotting	92
4.2.6.5.1. Protein transfer from the gel to the membrane	92
4.2.6.5.2. Immunodetection	92
4.2.6.5.2.1. Detection of the HRP conjugated secondary antibody-ECL	92
4.2.6.5.2.2. Detection of the AP conjugated secondary antibody	93
4.2.6.6. Anti-peptide antibodies	93
4.2.6.6.1. Synthesis of the peptides	93
4.2.6.6.2. Coupling of the peptides to a carrier protein	93
4.2.6.6.3. Immunisation	93
4.2.6.6.4. Purification of serum on peptide columns.....	93
4.2.6.7. Antibody against fusion protein.....	94
4.2.6.7.1. Preparation of <i>E. coli</i> cell lysate	94
4.2.6.7.2. Purification of fusion protein on Ni-column	94
4.2.6.7.3. Immunisation with fusion protein.....	95
4.2.6.7.4. Concentrating protein solutions	95
4.2.6.8. Determination of β -galactosidase activity (Ruan et al., 1993).....	95
4.3. Software	96
V. Summary	97
VI. References	100
VII. Appendices	107

I. Introduction

The myxobacteria have a remarkable life cycle that includes intercellular communication, cell differentiation and multicellular organisation. As a response to starvation myxobacterial cells undergo a specific developmental process leading to the formation of spores that are enclosed in fruiting bodies. Their development represents a model to investigate the flow of information between cells, signal transduction pathways and differential gene expression.

In higher organisms, complex morphological processes include differentiation of the cells from the same progeny into physiologically specialised tissues. Prokaryotic development includes changes in cell function and cell form in order to achieve benefit to the bacterial population to changes in environmental conditions. One aim of prokaryotic development is the formation of spores. In some bacterial species, as *Bacillus subtilis*, sporulation leads to the asymmetric division of the mother cell into two compartments (Piggot and Coote, 1976; Errington 1993). The small compartment called the forespore, matures to the metabolically quiescent endospore. The large compartment resembles the mother cell that lyses after spore maturation to set the spore free. In myxobacteria, differentiation of the vegetative cells into spores takes place at the end of the complex developmental cycle. As in higher organisms cell differentiation in myxobacteria is preceded by extensive cell movements and the formation of multicellular structures. In order to build up these multicellular structures myxobacterial cells coordinate their behaviour by intercellular signalling and direct cell-to-cell contacts.

The formation of fruiting bodies of myxobacteria shows great similarities to the life cycle of the cellular slime mould *Dictyostelium discoideum* (Raman, Hashimoto et al., 1976). During starvation, these unicellular amoebae form multicellular structures from which spores are formed that germinate when the conditions become more favourable.

The experimental accessibility of myxobacteria along with features mentioned above, represent them as a valuable prokaryotic model to study morphogenesis and development.

1.1. Myxobacteria

Myxobacteria are Gram-negative bacteria classified in the order *Myxococcales* that belongs into the delta-branch of the *Proteobacteria*. *Stigmatella aurantiaca* and the closely related *Myxococcus xanthus* are the best studied species of the myxobacterial group.

From the time of their detailed description by Ronald Thaxter in 1892 (Pfister, 1984) until now, myxobacteria fascinate scientist with their complex life cycle. The life cycle of the myxobacteria is bipartite. It is composed of a vegetative growth cycle and the developmental cycle, which is triggered by starvation. Upon nutrient depletion, the cells migrate into aggregations centres, from which fruiting bodies containing the myxospores arise. When nutrients become available, the myxospores germinate and the vegetative cycle starts again.

Myxobacteria grow on insoluble organic substrates such as decaying wood or leaves. Vegetative cells are rod shaped and about three times longer than *E.coli* cells. Since they do not have a flagella, the cells move by gliding, a special way of moving on a solid surface. Myxobacterial cells interact with each other forming a swarming community. The cells secrete slime containing lytic enzymes: lysozymes, proteases and also cellulases that degrade biopolymers. This way of feeding can be achieved only at high cells density, the so-called "wolf pack effect" (Dworkin, 1963).

Myxobacterial cells communicate with each other by direct cell-to-cell contact and by exchanging different signal molecules in the swarming community as well as during development. They represent so-called social prokaryotes.

Myxobacteria have a very large genome in comparison to other bacterial species (about two times larger than the genome of *E. coli*). The size of the *S. aurantiaca* genome is about 9,35 Mbp and is approximately equal to that of the myxobacterium *M. xanthus* (Chen et al., 1990; Neumann et al., 1992). The extremely large size of the myxobacterial genome reflects the potential to build multicellular structures during

development and the capability of these bacteria to produce a broad range of secondary metabolites (Schairer, 1993).

As a group myxobacteria produce a large spectrum of secondary metabolites like epothilon (Gerth et al., 1996), myxothiazol (Gerth et al., 1980), myxalamid (Gerth et al., 1996), stigmatellin (Kunze et al., 1984), soraphen (Gerth et al., 1994), TA (Rosenberg et al., 1973). Some of them are proven to be clinically very important.

1.2. Gliding motility

Myxobacteria move by gliding, a special form of locomotion that requires a solid surface (Burchard, 1984). Gliding cells move in the direction of their long axis, with stop intervals between and the reversal of the gliding direction. Many different classes of bacteria move by gliding. Recent studies suggest that bacterial gliding motility cannot be explained by only one model system. It is more likely that different types of motors are involved in gliding motility in different classes of bacteria. Some bacteria use type IV pilus extension and retraction powered by ATP hydrolysis to move over the surface (Merz et al., 2000). Gliding of some filamentous cyanobacteria depend on the polysaccharide extrusion (Hoiczky and Baumeister, 1998). Speculation about gliding in the myxoplasma group suggests involvement of the cytoskeleton and the surface adhesion proteins (Korolev et al., 1994; Lünsdorf and Schairer, 2001).

1.2.1. Gliding motility of myxobacteria

In *M. xanthus*, gliding motility is controlled by two separated multigene systems known as A (adventurous) and S (social) system (Hodgkin and Kaiser, 1979). The A system controls gliding of single cells, the S system is responsible for gliding of cells in groups. These two systems contribute equally to the wild-type gliding phenotype.

1.2.1.1. A system

The A system includes a minimum of 37 genes whose products control the interaction of the cell with the solid surface (Hodgkin and Kaider, 1979; MacNeil et al., 1994). Mutants defective in A motility are divided into two classes *cgl* (conditional gliding) and *agl* (adventurous gliding) (Hodgkin and Kaiser, 1979). There are several hypotheses about the mechanism of A motility.

One hypothesis suggests that import and export of macromolecules may be the direct force that move the cells, something like propulsion of the cells. This hypothesis is mostly based on the finding that the AgIU lipoprotein has similarities to the TolB protein of *E. coli* (White and Hartzell, 2000). The TolB protein is part of a large protein complex, which uses proton motive force to transport molecules across the outer membrane.

Another hypothesis suggests the involvement of some structures of the cell wall in A motility. The involvement of the specific surface structures in gliding was observed for the first time from the scanning electron micrographs of four different gliding bacteria species including *S. aurantiaca* and *M. xanthus* (Lünsdorf and Schairer, 2001). These structures are described as chain-like strands that associate with each other and form bands, which are wrapped, helically around the cell. The helical bands were not observed on the surface of cells treated with sodium azide or potassium cyanide. These two chemicals blocked the respiratory chain so that the cells were frozen and gliding motility was stopped.

1.2.1.2. S system

The S motility relies on the type IV pili. The S motility mutants lack polar pili and also removal of the pili from the wild type cells leads to defects in S motility (Kaiser, 1979). The *pil* gene cluster whose products are involved in pilus biogenesis are identified. PilA is the primary pilin protein, PilB is the putative NTPase functioning in pilus biogenesis, PilT is the putative NTPase acting in pilus retraction. PilG, -H and I are suggested to form an ATP-binding transporter involved in the transport of proteins

required in the pilus biogenesis (Wall et al., 1999; Wu et al., 1997; Wu et al., 1998). In addition to the *pil* genes, one other gene *tgl* (transient gliding) is required for S motility. Tlg is thought to be a lipoprotein whose function may be to facilitate pilus protrusion or pilus retraction (Rodríguez-Soto and Kaiser, 1997).

A model system that suggests the mechanism of the S motility proposes that pili are extended from the leading pole of the gliding cell. Contact between pili and surface induces pilus retraction, which results in cell movement (Kaiser, 2000; Sun et al., 2000).

1.2.1.3. The *mgl* locus

Another locus in *M. xanthus* with an important role in gliding motility is designated as *mgl* (mutual gliding). Mutations in the *mgl* locus abolish gliding motility of the cells. Two cotranscribed genes *mglA* and *mglB* have been identified (Stephens et al., 1989). The predicted amino acid sequence of MglA shows homology to the members of the GTP-binding protein class. MglA might have an important role to control expression of genes whose products are required for gliding motility (Hartzell, 1997). The predicted sequence of MglB exhibits similarities to one of the calcium binding sites of the yeast calmodulin (Hartzell and Kaiser, 1991).

The *S. aurantiaca mgl* genes were identified with a sequence homology of about 90% to the *mglA* and *mglB* genes of *M. xanthus*. Insertional mutagenesis showed that the *mgl* genes in *S. aurantiaca* are required for the motile phenotype of the cells (Schairer, 1993).

1.2.1.4. The *frz* locus

A genetic locus involved in the control of the frequency of reversal movements in *M. xanthus* is called 'frizzy' (*frz*). Cells with mutations in the *frz* genes either reverse direction much less frequently or much more frequently than wild type cells. The *frz* mutants showed impaired aggregation but produced normal spores. Six genes were identified *frzA*, -B, -CD, -E, -G and -F with homology to the chemotaxis genes of

flagellated enteric bacteria (She and Zusman, 1993; Ward and Zusman, 1997; Ward and Zusman, 1999).

1.2.1.5. Rippling

Rippling is a rhythmical movement of cells. The cells start to move synchronically to form a series of equidistant parallel ridges, which move in a pulsating manner. Myxobacteria appear to be the only procaryotes with this specific rhythmic behaviour (Reichenbach, 1986). Rippling precedes fruiting body formation but is not required for it. Rippling is induced by peptidoglycan. Thus, presence of rippling cells appears to be a sensitive indicator for the presence of extracellular peptidoglycan components. So it is more likely that rippling is incidental with fruiting body formation because rippling is induced by releasing peptidoglycan during development. Rippling requires the CsgA protein which is an extracellular polypeptide essential for *M. xanthus* development (Shimkets and Kaiser, 1982).

1.3. Fruiting body formation

As mentioned above, myxobacteria have a complex life cycle. During the vegetative growth phase, cells divide by transverse fission. Upon starvation, cells start to glide into aggregation centers from which the fruiting bodies arise. In the fruiting bodies vegetative cells differentiate into spherical, dormant myxospores. Each fruiting body encloses 10^5 myxospores, respectively. The shape of the fruiting body is species specific. Whereas *M. xanthus* fruiting bodies are simple mounds filled with spores, *S. aurantiaca* forms morphologically complex structures, resembling a small tree, with a branched stalk harbouring several sporangioles.

The morphological changes occur in a defined temporal order during development. In *S. aurantiaca* the whole process takes about 24 h. Different morphological stages during development are defined as early aggregates, early stalk (morel-like structure), late stalk (champignon-like structures) and mature fruiting bodies that are visible about 9, 12, 15 and 24 h after the beginning of starvation (Qualls et al., 1978a). Fruiting body

formation of *S. aurantiaca* is stimulated by incandescent light and requires the production of a pheromone (Qualls et al., 1978b).

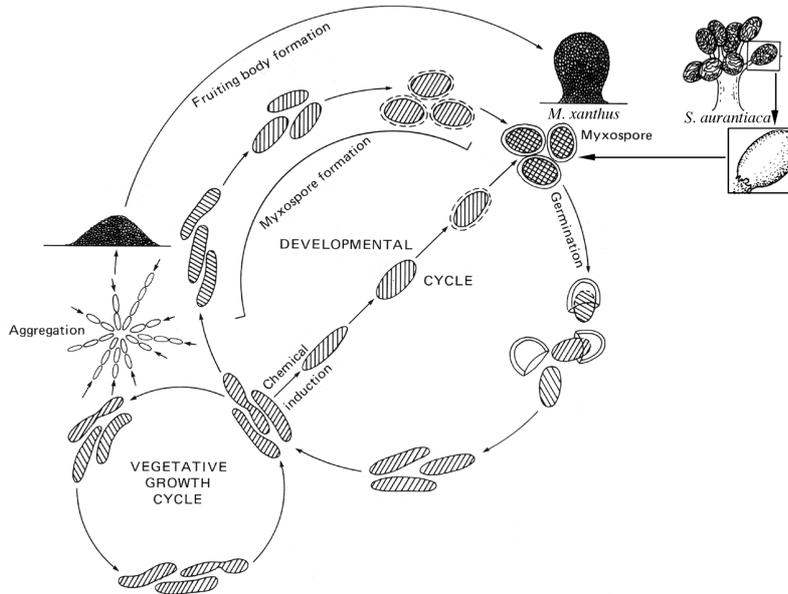


Fig.1.1. Diagram of the myxobacterial life cycle (Dworkin, 1985). Fruiting body of *M. xanthus* and *S. aurantiaca* are illustrated.

1.3.1. Pheromone activity in *S. aurantiaca*

S. aurantiaca cells secrete and respond to a pheromone that is necessary for fruiting body formation. The pheromone was eluted from cells assayed for fruiting body formation on filter paper and purified by steam distillation followed by reversed-phase and normal-phase HPLC (Plaga et al., 1998). It is a branched aliphatic hydroxy ketone, 2,5,8-trimethyl-8-hydroxy-nonan-4-one, named stigmolone (Hull et al., 1998). Stigmolone is a new type of a pheromone molecule in prokaryotes since elucidation of its chemical structure showed that it does not belong to any known class of pheromones up to now. It acts in concentrations of about 1 nM to shorten the time of aggregation on the beginning of development in a bioassay (Plaga et al., 1998). Addition of purified stigmolone accelerated the rate of aggregation when added to 5×10^7 cells. The aggregation rate was comparable to that observed in a population of 2×10^8 cells as

control without addition of the pheromone (Plaga, et al., 1998). Stigmolone activity correlates with the number of cells. Therefore, stigmolone may have a role in “quorum sensing” at the beginning of the developmental cycle. Species-specificity is indicated by the fact that *M. xanthus* does not respond to stigmolone by accelerating fruiting body formation in a bioassay (Plaga et al., 1998).

Cells have to be in contact with each other or with a solid surface to secrete stigmolone. The stigmolone biosynthetic pathway and the putative pheromone receptor are still unknown. The structure of the stigmolone suggests the involvement of some metabolites from the catabolism of leucine or from a biosynthetic pathway leading to terpenoids, fatty acids or polyketides (Plaga et al., 1998).

1.3.2. Artificially induced sporulation

Sporulation can be induced independently from fruiting body formation by addition of various chemicals. In *S. aurantiaca* indol and some indol derivatives are the most potent inducers of sporulation (Dworkin, 1994; Gerth and Reichenbach, 1978).

The starvation dependent and starvation independent sporulation follows a time scale and has different nutritional requirements and different inducers. There are also structural differences between the two types of spores. The starvation-independent spores of *M. xanthus* lack the fruiting body spore protein S (Komano et al., 1980), the coat is thinner (Zusman, 1980) and they contain more ribosomes. Both kinds of spores contain protein U (Komano et al., 1980) and both pathways of sporulation induce a beta-lactamase activity (O'Connor and Zusman, 1997).

1.3.3. Genes involved in *S. aurantiaca* fruiting body formation

To identify developmentally regulated genes in *S. aurantiaca* Tn5lacZ transposon mutagenesis was performed (Pospiech et al., 1993). Three different classes of mutants impaired in fruiting body formation were detected. Members of the first class form abnormal fruiting bodies, those of the second-class aggregate into clumps, and those of the third class of mutants are not able to aggregate at all (Pospiech et al., 1993).

Further analysis of the transposon induced mutant AP182 led to the identification of the *fbfB* gene involved in fruiting. Analysis of the upstream and downstream regions of *fbfB* showed the existence of further *fbf* genes, *fbfA*, *fbfC*, *fbfD* (Müller, 2002; Silakowski et al., 1996) that are arranged in the same orientation. *fbfC* and *fbfD* form an operon and more or less, the whole *fbfA* sequence is needed for the correct expression of *fbfCD*. The gene *fbfB* is located upstream of *fbfA* in a divergent orientation (Silakowski et al., 1998). The gene product of *fbfA* shows a homology of about 30% to the *N*-acetylglucosamine transferase (NodC) of *Rhizobium meliloti* and the chitin synthase of *Saccharomyces cerevisiae*. These enzymes are involved in the synthesis of extracellular polysaccharides. FbfA therefore might be an enzyme catalyzing the synthesis of extracellular polysaccharides that are involved in signalling. *FbfB* encodes a putative protein that shows homology to the galactose oxidase of *Dactylium dendroides* (Silakowski et al., 1998). A putative function of FbfB could be the oxidation of primary alcohols to aldehydes (Silakowski et al., 1998). The putative FbfC polypeptide has no homology to known proteins. FbfD shows homology to an ORF with unknown function of *M. xanthus* (Müller, 2002). Insertion of the *neo* gene into each of the *fbf* genes led to mutants that form just clumps during starvation. Mixing of *fbfA* mutant cells with the nonaggregating transposon mutant AP191 led to a partial phenotypic complementation, the formation of a morel-like structure (Silakowski et al., 1996). Mixing of the *fbfB* mutant cells with AP191 led to the formation of a champignon-like structure.

Analysis of the *fbf* gene expression in merodiploid strains containing various large upstream regions of the analysed *fbf* gene 3'truncated and fused to the \square *trpA-lacZ* reporter gene revealed that each gene from the cluster is expressed during development. The *fbfA* gene is transcribed about 8 h after the start of development, *fbfB* is expressed about 14 h after induction of the fruiting body formation. The genes *fbfC* and *fbfD* are both expressed about 8 h after induction of starvation. Downstream of the *fbfB* gene the *mta* gene cluster was detected encoding polyketide synthases and nonribosomal peptide synthetases. These two *mta* cluster products are involved in the synthesis and modification of the secondary metabolite myxothiazol and not in fruiting body formation (Silakowski et al., 1998; Silakowski et al., 1999).

1.4. Intercellular signalling and communication in bacteria

Bacteria use sophisticated chemical communication systems in order to coordinate the behaviour of their populations. This capability is important to improve access to different nutrient sources, to achieve rapid colonisation of a new ecological niche. Bacterial communication also allows survival of the population by differentiation into morphologically more resistant forms or defence against competitive microorganisms or the eukaryotic immune system (Shapiro, 1988). Bacterial cells are able to respond to different molecules produced by bacteria but also by plants or animals cells, and the other way round. Cell density dependent conjugal transfer of Ti plasmids between *Agrobacterium tumefaciens* cells is triggered by opines produced by the plant host (Zhang et al., 1993). The homoserine lactone which is the density sensing molecule in *Pseudomonas aeruginosa* can also influence the host immune response (Telford et al., 1998). Some pathogenic bacterial species produce molecules that can bind to hormone receptors and in this way bacteria may manipulate eukaryotic host cell signal transduction pathways.

Information transfer between cells determines differentiation and morphogenesis in a wide variety of bacterial systems: induction of luminescence in *Vibrio* by homoserine lactones, sporulation in *Bacillus*, erection of aerial hyphae by *Streptomyces*, fruiting body formation in myxobacteria.

Signalling molecules can be small diffusible molecules and secreted polypeptides as well as surface associated macromolecules. They are also called bacterial "hormones", "pheromones" or "autoinducers" (Wirth et al., 1996). Bacteria use signalling molecules to monitor the state of other cells in the population. The so-called "quorum sensing", a cell density sensing mechanism, enables bacteria to function as multicellular organisms. This cell density sensing mechanism depends on the activation of a response regulator by a self-generated diffusible signal molecule.

1.4.1. Intercellular signalling in Gram-negative bacteria

Gram-negative bacteria use homoserine lactones as small signalling molecules that diffuse across the outer and inner membrane to reach their target protein in the cytoplasm. One of the first described autoinducers was the N-acyl-homoserine-lactone autoinducer (AHL) from the marine bacterium *Vibrio fischeri* involved in the control of bioluminescence. Luminescence operons consist of several genes (Engebrecht et al., 1983). The *luxR* gene encodes an autoinducer dependent transcriptional activator of the *luxI-G* operon. LuxI is an autoinducer synthase. LuxC, D and E form a complex that generates long-chain fatty aldehyde, actual substrates of the luciferase reaction. LuxA and B are two subunits of the luciferase. The function of LuxG is unknown. Cellular and environmental concentration of this signal molecule (AHL) is identical, since it diffuses freely through membrane (Kaplan and Greenberg, 1985). At high cell densities the concentration of the autoinducer increases and reaches a sufficient high concentration to bind to LuxR. LuxR in turn activates the transcription of the *lux* operon.

Beside the homoserine lactone mediated "quorum sensing" other molecules with signalling function have been identified. The gamma-butyrolactones in *Streptomyces* are involved in the control of antibiotic biosynthesis, resistance and differentiation (Horinouchi and Beppu, 1992). Butyrolactones have antifungal activity in *Pseudomonas aureofaciens* (Gamard et al., 1997) and the 3-hydroxypalmitic acid methyl ester is involved in regulation of virulence in the plant pathogen *Ralstonia solanacearum* (Flavier et al., 1997).

1.4.2. Intercellular signalling in Gram-positive bacteria

Gram-positive bacteria, use small modified peptides as signalling molecules that can interact with two-component histidine kinase signal transduction systems (Wirth et al., 1996). Small octapeptides act as signalling molecules to regulate cell density dependent virulence gene expression in *Staphylococcus aureus* (Ji et al., 1995). Extracellular signal peptides are involved in the initiation of sporulation in *Bacillus subtilis*.

Heptadecapeptides are necessary for the regulation of genetic competence in *Streptococcus pneumoniae* (Pestova et al., 1996).

1.4.3. Intercellular signalling in *M. xanthus*

Myxobacterial development strictly depends on signalling between cells. These signals coordinate temporal gene expression in the course of development. The cell-cell signal mutants are unable to complete development by themselves, but they can overcome this developmental block when they are mixed with wild type cells. The result of the complementation studies indicate that cell-cell signalling mutants can be placed into several different classes. Mutants from the same class fail to complement each other. Mutant cells are defective in producing a signal but they retain the ability to respond to the signal (Hagen et al., 1978; Janssen and Dworkin, 1985; LaRossa et al., 1983; Shimkets and Dworkin, 1981). At least five different signalling pathways have been identified in *M. xanthus*.

1.4.3.1. A signalling

Mutants defective in producing the A signal arrest at about 1 to 2 hours after initiation of development in the preaggregation stage as a flat film of cells. Five genes known as *asgA*, *asgB*, *asgC* (Kuspa and Kaiser, 1989; Plamann et al., 1994; Plamann et al., 1995; Shimkets, 1999), *asgD* (Cho and Zusman, 1999) and *asgE* (Garrza et al., 2000) have been identified to function together in order to produce the active A signal. The A signal is proposed to be a mixture of amino acids and peptides generated in amounts proportional to the cell density by extracellular proteolysis (Kuspa et al, 1992; Plamann et al., 1992).

1.4.3.2. B signalling

The B signal acts early in development. All of the *bsg* mutations fall into one single gene named *bsgA* (Gill and Cull, 1986). Mutants fail to aggregate, sporulate, and are unable to express developmentally regulated genes. The *bsgA* gene product is an ATP-dependent protease with homology to the Lon protease of *E. coli* (Gill et al., 1993). The

suggested role of the BsgA protease is that it is involved in the regulation of the initiation of the developmental phase.

1.4.3.3. C signalling

The C signalling pathway is the most intensively studied in *M. xanthus*. The C signal acts about 6 h after the beginning of development. All *csg* mutations fall into a single genetic locus named *csgA*, formerly known as *spoC* (Shimkets et al., 1983). Mutants unable to synthesized CsgA fail to ripple, aggregation and sporulation are severely impaired and expression of developmental genes that is normally induced 6 hours after the beginning of starvation is reduced or abolished (Shimkets et al., 1983; Kroos and Kaiser, 1987). Overproduction of CsgA leads to premature aggregation and sporulation as well to the formation of small fruiting bodies (Kruse et al., 2001). In contrast, reduced synthesis of CsgA causes a delay in aggregation, reduces the ability to sporulate and causes the formation of large fruiting bodies (Kruse et al., 2001). *csgA* expression slowly increases during development and reaches a peak at the sporulation stage (Hagen and Shimkets, 1990).

The *csgA* mutant phenotype can be restored by adding the purified CsgA from immature wild type fruiting bodies or the MalE-CsgA fusion protein produced in *E. coli* (Kim and Kaiser, 1990; Lee et al., 1995).

The predicted amino acid sequence of CsgA shows homology to the members of the short-chain alcohol dehydrogenases family. These enzymes use NAD(H) or NADP(H) to catalyze the interconversion of secondary alcohols and ketones or mediate decarboxylation (Persson et al, 1991). The CsgA protein with a single mutation at the N terminus was unable to bind radiolabeled NAD⁺ *in vitro* and to rescue the *csgA* mutant phenotype (Lee et al., 1995). A single amino acid substitution in the putative substrate binding domain of the CsgA protein leads to a mutant unable to develop (Lee, et al., 1995).

The CsgA is an extracellular protein associated with cell surface (Kim and Kaiser, 1990; Shimkets and Rafiee, 1990). It is still unclear if CsgA has an extracellular

enzymatic function to convert some substrates into the chemically active C signal or whether CsgA acts as a signal itself.

The reported importance of the putative coenzyme binding site and the putative substrate binding site for CsgA function support the first model (Lee, et al., 1995). Additionally, overproduction of SocE, another member of the short-chain alcohol dehydrogenase family, rescues the developmental phenotype in the *csgA* mutant (Crawford and Shimkets, 2000). The putative CsgA substrate is unknown and it is hard to predict the structure of the substrate since members of the short alcohol dehydrogenase family have a large spectrum of substrates. The second model is based on the findings that the 17 kDa protein isolated from wild type cells during development can restore the *csgA* mutant phenotype (Kim and Kaiser, 1990). Two forms of the CsgA protein have been identified in extracts of developmental cells. A large form of 25 kDa that corresponds to the full-length protein encoded by the *csgA* gene and a smaller form of 17 kDa (Kruse et al., 2001). The large form might represent a precursor protein that becomes proteolytically cleaved to a polypeptide of 17 kDa that has C signalling activity.

Despite unclear nature of the C signal, the cellular responses to C signalling are known. As mentioned above it induces rippling, aggregation, sporulation and expression of many genes including *csgA* itself. It was shown by addition of the purified 17 kDa protein to *csgA* mutant cells (Kim and Kaiser, 1990) and by reducing the transcription of *csgA in vivo* by nested deletions of the upstream region (Li et al., 1992) that a low concentration of the C signal is required for rippling and aggregation. A higher concentration induces sporulation and C signal dependent gene expression including *csgA* itself. Therefore the model of the C signalling pathway indicates two branches. One branch leads to the regulation of the movement responds of cells and the other branch controls sporulation and expression of the late developmental genes (Fig.1.2.).

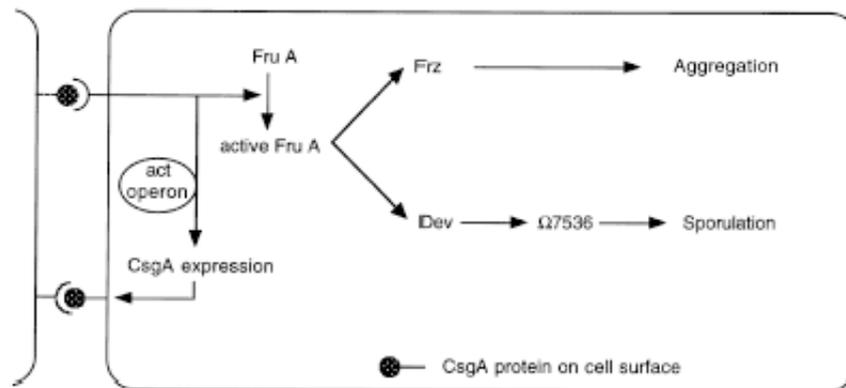


Fig. 1.2. A model for the C-signal transduction pathway (Gronewold and Kaiser, 2001).

Upstream in the C signalling pathway, the *act* operon controls the level and the time course of CsgA production (Gronewold and Kaiser, 2001). The CsgA activates FruA, which is a transcriptional regulator protein with a putative helix-turn-helix DNA binding domain (Ellehaug et al., 1998). Synthesis of FruA is regulated on the transcriptional level and does not depend on C signal (Ellehaug et al., 1998). More likely C signal transmission induces activation of FruA presumably by phosphorylation (Ellehaug et al., 1998). Downstream from FruA the C signal pathway branches. One branch leads to the regulation of rippling and aggregation. Active FruA induces methylation of FrzCD protein, a homolog to bacterial chemotaxis proteins. Frz proteins are important for rippling and aggregation and control of specific gliding parameters in response to the C signal (Zusman 1982; Jelsbak and Sogaard-Andersen, 1999). Increased methylation of the FrzCD correlates with a decreased reversal frequency, allowing the cells to move in chains into the aggregation center. When cells aggregate, the level of C-signal increases. In the presence of high level of a C signal and active FruA, the *dev* operon and other late developmental genes are expressed which are required for sporulation (Thöny-Meyer and Kaiser, 1993).

1.4.3.4. D signalling

The D signal acts between 1 and 2 h after beginning of development. Only one gene is identified, designated as *dsgA* (Chang and Kaiser, 1989). Mutations in *dsgA* delay aggregation and reduce the sporulation efficiency. The *dsgA* gene encodes a protein with 50 % similarity to the translation initiation factor IF3 of *E. coli* (Chang and Dworkin, 1994).

1.4.3.5. E signalling

The E signal acts about 3-5 h after the beginning of development. The E signal mutant fails to aggregate or sporulate normally. Two genes were identified *esgA* and *esgB* that encode the E1 α and E1 β subunits of the branched-chain α -keto acid dehydrogenases involved in amino acid and fatty acid metabolism (Downard and Toal, 1995; Toal et al., 1995). Downard and Toal (1995) assumed that long branched-chain keto acids are incorporated into the phospholipid membrane during vegetative growth. During development they are released and act as the actual E signal.

1.4.4. Intercellular signalling in *S. aurantiaca*

S. aurantiaca forms morphologically more complex fruiting bodies in comparison to *M. xanthus* and therefore represents a better prokaryotic model to study genetic determination of morphogenesis. Formation of the complex fruiting bodies of *S. aurantiaca* consist of a branched stalk with sporangioles requires specific communication between the cells in order to coordinate their behaviour. One type of the signalling molecule was isolated from *S. aurantiaca* cells (Plaga et al., 1998). As mentioned above it is a novel type of pheromone, which acts to help cells to stay together in the aggregation phase.

Another gene product involved in intercellular signalling was identified in *S. aurantiaca*, the *csgA* homolog of *M. xanthus*. Inactivation of the *csgA* gene in *S. aurantiaca* was reported to affect fruiting body formation (Butterfass, 1992).

1.5. The aims of this work

As mentioned above *S. aurantiaca* is a social prokaryote. Cells communicate with each other by direct contact or by exchanging various signal molecules. Isolation and characterization of these signalling molecules would contribute to the understanding of the complex life cycle of *S. aurantiaca*.

The *M. xanthus* *csgA* gene homolog was identified in *S. aurantiaca*. Inactivation of the gene was shown to impair fruiting body formation. Because of the strong evidences supporting the role of the CsgA protein in intracellular signalling in *M. xanthus*, an initial characterisation of the *csgA* gene in *S. aurantiaca* was undertaken.

The work of this thesis includes cloning of a DNA fragment harbouring the *csgA* gene flanked by its upstream and downstream sequences; a detailed sequence analysis of the *csgA* upstream region; elucidation of the CsgA function *in vivo* by disrupting the *csgA* gene and observation of the mutant phenotype during starvation; investigation of *csgA* transcription in a merodiploid strain that contains the upstream region of *csgA* fused to a *lacZ* reporter gene; immunological identification of the CsgA protein during *S. aurantiaca* growth and development.

II. Results

2.1. Molecular cloning and sequence analysis of the *csgA* locus from *S. aurantiaca*

In *M. xanthus* the *csgA* encoded polypeptide is a molecular timer for the developmental program. It constitutes the C signalling pathway that is required for regulation of the correct temporal order of the three morphological stages known as rippling, aggregation and sporulation during development. It is also involved in the regulation of the expression of developmental genes that are expressed about 6 h after the beginning of starvation. According to the important role of CsgA in the *M. xanthus* development and because of the close phylogenetic relation to *S. aurantiaca*, the question arised if there is a similar C signalling pathway in *S. aurantiaca* involved in intercellular signalling during fruiting body formation.

To address this question it was first necessary to identify the homologous gene in *S. aurantiaca*. Previously the *S. aurantiaca csgA* homolog was identified by Southern blot analysis using a *M. xanthus csgA* gene probe. An *EcoRI* fragment (12 kbp) harbouring the *csgA* gene was subcloned from a *S. aurantiaca* lambda gene library into the plasmid pUC18. Sequence analysis revealed that an *EcoRI* fragment contained the coding part of the *csgA* gene flanked by a 148 bp upstream sequence. The *S. aurantiaca csgA* gene sequence was added into the EMBL/GenBank data base, accession number M95300 (Butterfass, 1992). The *csgA* gene encodes a protein of 173 amino acids with a predicted molecular mass of about 19 kDa. The gene sequence shows about 70 % homology to the *M. xanthus csgA* gene. The deduced amino acid sequence of the *S. aurantiaca csgA* revealed an identity of about 54% to the *M. xanthus CsgA*.

A *csgA S. aurantiaca* mutant phenotype was reported previously (Butterfass, 1992). A merodiploid mutant strain was constructed in which 2 truncated copies of the gene were present, separated by vector sequences. One *csgA* allele was lacking the 3' part and another *csgA* allele was disrupted by the insertion of a kanamycin cassette, so that the merodiploid mutant did not contain a functional *csgA* gene. Under starvation conditions this mutant formed a bulk of sporangioles without a differentiated stalk and a delay of 12 hours in the time course of the mutant development as compared to the wild type was reported (Butterfass, 1992).

2.1.1. Cloning of the *csgA* locus from *S. aurantiaca*

On the basis of this data, the putative role of the *csgA* gene product during *S. aurantiaca* development should be analysed in detail. To characterize *csgA* expression during development and to construct a *csgA* insertional mutant strain (double recombination) it was first necessary to isolate a larger DNA fragment harbouring the *csgA* coding region flanked by the whole upstream and by long downstream sequences.

A 465 bp internal fragment of the *csgA* gene was amplified by PCR using the chromosomal DNA of the *S. aurantiaca* wild type as a template and the primers *csgA* 7/*csgA* 8. The purified PCR product was biotin labelled and used as a probe in a Southern analysis of *S. aurantiaca* wild type DNA digested with various restriction enzymes. The *SalI*-, *XbaI*-, *NotI*-, *HindIII*-, *SacI*- chromosomal DNA fragments which hybridized with the probe were too large (more than 7,7 kbp) for subcloning in standard cloning vectors (Fig.2.1.). Restriction with *BamHI* resulted in a fragment of about 1,7 kbp which was not large enough to contain the upstream and downstream regions of the *csgA* gene. A 3 kbp *XhoI* fragment that hybridized with the probe had a suitable size for further cloning.

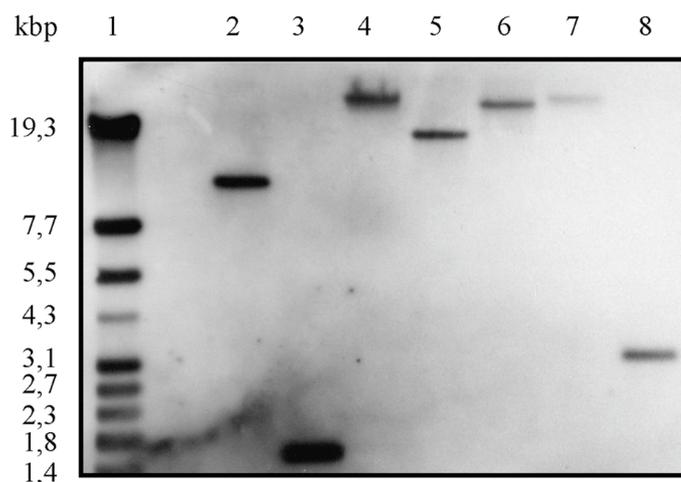


Fig.2.1. Southern analysis of restricted chromosomal DNA of the *S.aurantiaca* wild type using the biotin labelled *S. aurantiaca csgA* gene as a probe. 10 μ g of DNA (lanes: 2-8) were digested with *SalI*, *BamHI*, *XbaI*, *NotI*, *HindIII*, *SacI*, *XhoI* and separated on a 0,9 % agarose gel. The size of the fragments were estimated using the DNA Molecular Weight Marker IV (Roche Diagnostics)-lane 1.

2.1.1.1. Preparation and screening of a *S. aurantiaca* genomic library

In order to facilitate cloning of *S. aurantiaca* *csgA* gene locus, a genomic library was constructed. The bacteriophage vector Lambda DASH II (Stratagene) predigested with restriction enzyme *Bam*HI, which accomodates inserts ranging from 9 to 23 kbp, was used as the vector to construct the genomic library of *S. aurantiaca*. To obtaine a truly random library, high molecular weight chromosomal DNA from *S. aurantiaca* was fractionated by partial digestion with the restriction endonuclease *Sau*3AI which produces *Bam*HI-compatible cohesive ends. Serial dilutions of *Sau*3AI were prepared to determine the optimal concentration of the enzyme necessary for generating DNA fragments with a size of 9 to 23 kbp. Partial digestion was accomplished using 0,025 U of *Sau*3AI per microgram of chromosomal DNA in a reaction incubated at 37°C for 40 min. Obtained DNA fragments were cut out from the gel and purified with the gel extraction kit (Qiagen). Library construction was performed following the instruction manual of lambda DASH II/ *Bam*HI vector kit (Stratagene) and the detailed steps are described in Materials and Methods. The size of the constructed genomic library was determined to be 1×10^5 plaque forming unit (pfu)/ μ g of genomic DNA, which offers more than 99% probability of isolating a particular gene from a bacterial genome.

The 465 bp *csgA* PCR fragment from the *S. aurantiaca* wild type (see 2.1.1) was used as biotin labeled probe to screen the *S. aurantiaca* phage library. Six independent plaques that hybridized with the probe were detected. The DNA of the positive phages was isolated and Southern analysis was performed using the same probe as for the screening of the library. One positive clone designated λ 11, respectively, was chosen for further work. In the Southern analysis (Fig.2.2.) of *Xho*I digested λ 11 DNA one fragment of about 3 kbp was detected. Double digestion with *Xho*I/*Eco*RI revealed one fragment of about 1,5 kbp which hybridized with the *csgA* probe. According to previous data, λ 11 probably harboures about 1,5 kbp of the upstream region of *csgA*. Digestion of lambda 11 with *Sal*I revealed a fragment of about 11 kbp that hybridized with the probe.

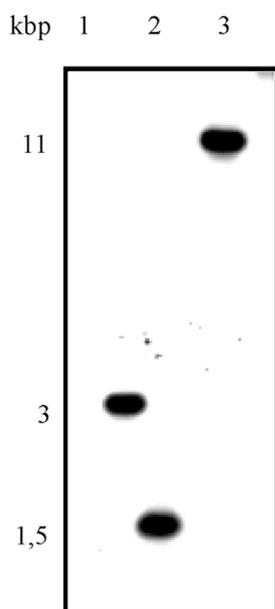


Fig.2.2. Southern analysis of restricted ϕ 11 phage DNA using the *csgA* gene as a probe. Lane 1-2: ϕ 11 DNA (2 μ g) digested with *Xho*I; *Xho*I/*Eco*RI and *Sal*I separated on 0,9 % agarose gel. The size of the fragments was estimated using DNA Molecular Weight Marker IV (Roche Diagnostics).

2.1.1.2. Subcloning of the 3 kbp *Xho*I fragment harbouring the *S. aurantiaca csgA* gene

The 3 kbp *Xho*I fragment harbouring the *csgA* gene was purified from ϕ 11 phage DNA, restricted with *Xho*I and separated on a 0,8 % low-melting agarose gel using the gel extraction kit (Qiagen). Isolated *csgA* fragment was used in subsequent ligation reactions with different vectors: Litmus 28, pBSSK-, pBCSK- and pUC18, pre-digested with *Xho*I. Ligation reactions were used for the transformation of *E. coli* DH5 α . After white-blue screening and restrictions analysis positive transformants were not identified.

Since subcloning of the *Xho*I fragment in the previously described vectors did not leads to success when the *E. coli* DH5 α host strain was used, therefore special restriction minus *E. coli* competent cells, XL1 - Blue MRF', which make possible the cloning of the highly methylated DNA were used in subsequent transformation reactions. Clones

obtained after the transformations were restricted with *Xho*I but only religated plasmids were detected.

Another special *E. coli* strains ABLE C and ABLE K (Stratagene) in which the copy number of cloning vectors per cell, is reduced thus increasing the probability to clone DNA encoding toxic proteins, were used as host cells. Again no positive transformants were detected by restriction analysis.

In total, more than three hundred different clones were screened by restriction analysis. Therefore it was concluded that cloning of the *Xho*I *csgA* fragment from *S. aurantiaca* was not possible in high or medium -copy number plasmids.

2.1.1.3. Subcloning of the *Xho*I *csgA* fragment from □11 into the plasmid pACYC 177

Final approach for subcloning of the *Xho*I fragment from □11 the low copy number vector pACYC 177 (New England Biolabs) was used. The 3 kbp *csgA* fragment from □11 was purified as previously described and inserted into pACYC 177 digested with *Xho*I. The recombinant plasmid was used to transform *E. coli* XL1-Blue MRF⁺. The insertion of DNA fragment into the *Xho*I site of the plasmid pACYC 177 leads to a disruption of the *neo* gene. This was used for screening of positive clones by replica plating. A total of 192 clones were transferred after growth in LB medium supplemented with tetracycline and ampicillin on LB plates containing additionally kanamycin. Only clones which were not able to grow on LB plates with kanamycin were further analysed. One plasmid designated pAM5 showed a correct restriction pattern after digestion with *Xho*I. Southern analysis of the *Xho*I digested pAM5 with biotin labelled PCR product of the *csgA* gene of *S. aurantiaca* revealed a fragment of about 3 kbp.

2.1.2. Sequence analysis of the *csgA* locus from *S. aurantiaca*

The nucleotide sequences of both strands of the 3 kbp *Xho*I fragment were determined and analysed further. The sequence data confirmed that the *Xho*I fragment

harbours the complete sequence of the *csgA* gene and additionally the upstream and downstream region with approximately the same size. Sequences data were compared with those already published (Butterfass, 1992) and no differences were found.

As previously reported (Butterfass, 1992) a 840 bp fragment harbouring *csgA* gene was sequenced. Sequence analysis revealed a GTG codon at position 148 as the putative start codon and a TAG codon at position 679 as stop codon of the *csgA* gene. No Shine-Dalgarno sequence was found in front of the putative start codon. The gene encodes a protein of about 173 amino acids with a calculated molecular mass of about 19 kDa.

Isolation and sequencing of the much larger fragment of 3 kbp harbouring the *csgA* gene made it possible to further analyse 1,5 kbp of the *csgA* upstream sequence.

Upstream from the proposed *csgA* start codon GTG (located at position 1533 on the 3 kbp *XhoI* fragment) two additional putative start codons were found in the correct reading frame (Fig.2.3.). The first ATG codon (located at position 1185) is preceded by a Shine-Dalgarno sequence (GGAGG) in an unfavourable distance from the start codon (2 bp from ATG). The second ATG codon (located at position 1344) has no Shine-Dalgarno sequence. The size of the *csgA* gene starting with a ATG (bp 1185) is 870 bp and encodes a putative polypeptide of 289 amino acids with a calculated molecular mass of about 32 kDa. The size of the gene starting with the second ATG (bp 1344) is 711 bp long and encodes a putative polypeptide of 236 amino acids with predicted molecular mass of about 26 kDa. Those two putative start codons located upstream of the first proposed GTG start codon cannot be excluded as initiation sites of the *csgA*-encoded protein.

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TCACCCC6CCCAGGT666CACCGGCTTCCAGCACGACCGCATCCTTTCCACGTGAG6C66A66CGATGCCCAGC6C6AGC 1200
                                     M R Q R E
CC6GTGATGCCTCCTCCGACGACGGCAATGACGGCCATAACCGTTACTCCTGG6TCTGCGTGCCCTCCCTCCTTCGAG6C 1280
P G D A S S D D G N D G H N R Y S W V C V P S L L R G
TG6CGCG66GTCAAGCATCACATGG6TTGTGCCTACAGCGTGCAGCGCGGTTAATACACCCATATGCGCTACGCCATCTC 1360
W R A G Q A S H G L C L Q R A T R V N T P M R Y A I S
CG6TGCCAGCCGTGGCATCG6TCTCGAATTCGTCCGGCAACTTCTCGAACGTGGCGACACTGTGGAG6C6666GTCCGAG 1440
G A S R G I G L E F V R Q L L E R G D T V E A G V R
TTCCACG6AG6CCCG6TTGCTCTCCCCGCTGATGAACAGCGTGC6GCCCTCGCTGCG6ATTACGAGCTCGACATCACC 1520
V P T E A R L L S P L M N S V G P R L R I H E L D I T
AACCAG6CCAGTGTGCGTGCCCTTCGCTCG6CCGTGAGCGACGGTCCGCTGGATGTGCTCATCAACAACGCGAG66GTCA6 1600
N Q A S V R A F A S A V S D G P L D V L I N N A G V S
CG6CAAGTGGTGCCTTCATGGAGATGGACTACGAGGACATGACGAAG6TTCATGGAGACCAACTCCGTGG6CCCCATGC 1680
G K W C S F M E M D Y E D M T K V M E T N S V G P M
GTCTGTCCGCGCGCTGATGCCCG6GTGCTCAAG66CCCGACGCG6AAGATCATCCACCTGACCACGCGCATGGCCTCG 1760
R L S A A L M P A V L K G P T R K I I H L T T R M A S
CTCACGGAGAACACCC666CG6GTCTATGG6TTCCAG66CG666CATATGCCTACCGCATGTGCAAG6CCGCGTTGAA 1840
L T E N T R G G V Y G F E G G A Y A Y R M S K A A L N
CGTGTGCATGCGCACCATGGCCGTGGACTTCCGG6ACCA666GCTCATCACC6CG6GATCAACCC666GTGG6TGC6CA 1920
V C M R T M A V D F R D Q G L I T A A I N P G W V R
CG6AGATGG6CG6CAAGCTGG6CGCGATGCGTCCAGAG6ACGCCGTCC6666GATGTTGCGCGTCATCGATGACATCACC 2000
T E M G G K L A P M R P E D A V R G M L R V I D D I T
AAGGAGCAGAGCG6CATGTTCTCGACTTCCAG66CG6GAG6TGCCTGGTAG66CAG6666CATGG6CG6GCTCAG6 2080
K E Q S G M F L D F Q G R E V P W .

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Fig.2.3. Nucleotide sequence of the *csgA* locus (part of the 3kbp sequence from 1120 bp to 2080 bp is shown). Putative start codons ATG (1185 bp), ATG (1344 bp) and GTG (1533) of the *csgA* gene (are underlined) and deduced amino acid sequences of CsgA protein. The serine, tyrosine and lysine residues in the putative catalytic site of CsgA protein are underlined.

The alignment of the *S. aurantiaca csgA* gene with *M. xanthus csgA* is shown in Fig. 2.4. All three putative start codons of the *S. aurantiaca csgA* were indicated on the alignment report. The comparison of the three possible *S. aurantiaca csgA* genes with the *csgA* sequence of *M. xanthus* was performed using the program Multiple Sequence Alignment of the Lasergene program package. The alignment revealed a homology of about 63% when *S. aurantiaca csgA* translation start codon is ATG located at position

1185 or at position 1344. The homology between the previously proposed *csgA* coding sequence of *S. aurantiaca* (Butterfass, 1992) and *M. xanthus* is about 61%.

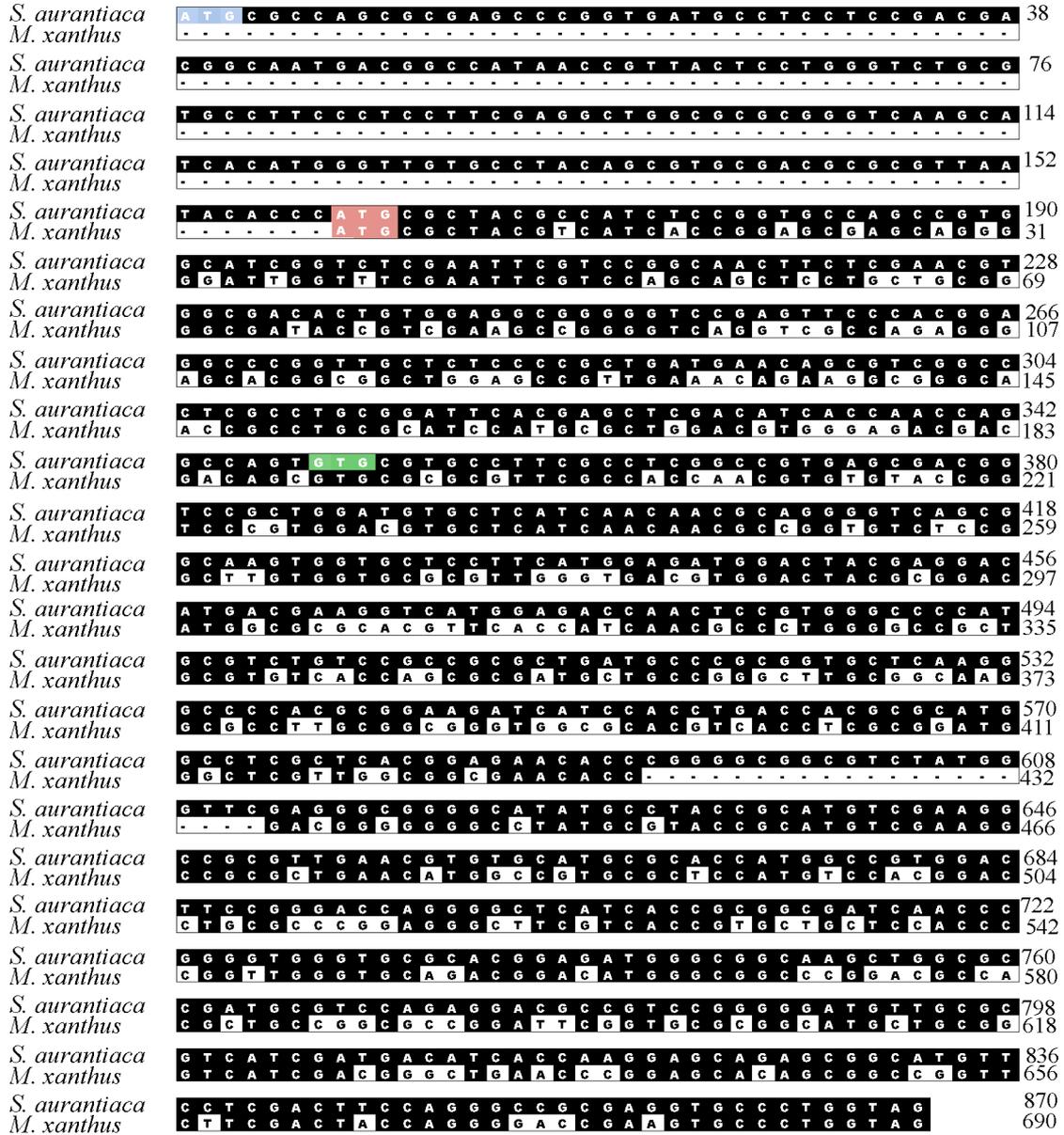


Fig.2.4. Alignment of the *S. aurantiaca csgA* gene that of *M. xanthus*. The putative start codons of the *S. aurantiaca csgA* are shown in coloured boxes: potential start codon ATG (bp 1185) is shown in a blue box; potential start codon ATG (bp 1344) is shown in a red box; proposed start codon GTG (bp 1534) is shown in a green box. The start codon of the *M. xanthus csgA* gene is shown in a red box.

The alignment of the deduced amino acid sequence of the *S. aurantiaca csgA* coding region and the amino acid sequence of the CsgA from *M. xanthus* is shown in Fig.2.5.

2.1.2.1. Analysis of the upstream and downstream sequences of *csgA*

Analysis of the sequences flanking *csgA* revealed three putative ORFs. The analysis was performed by setting up the minimal number of amino acids for the ORFs to 170. Two of these ORFs were identified upstream and one downstream of *csgA* (Fig.2.6.). A part of the ORF1 (bp 1-1102) with a putative start codon ATG and no Shine-Dalgarno sequence is located upstream of *csgA* in a divergent orientation. The stop codon of ORF1 could not be identified on the *XhoI* fragment. The nucleotide sequence shows a homology of about 60% to a gene that codes for a *protoporphyrinogen oxidase* in *M. xanthus*. Due to the high similarity ORF1 was designated as *protoporphyrinogen oxidase* gene. ORF2 (bp 350-1093) overlaps with the 5' part of the *protoporphyrinogen oxidase* and encodes a putative polypeptide of 247 amino acids (ca 27 kDa). No suitable Shine-Dalgarno sequence was found in front of the putative start codon ATG. However, no sequence has been found to be homologous to the deduced amino acid sequence of ORF2. The third open reading frame, ORF3, (bp 1996-2667) is located downstream from *csgA* in a divergent orientation. It codes for a polypeptide of 223 amino acids (ca 25 kDa). The 3' part of ORF3 (55 bp) overlaps with the 3' terminus of *csgA*. The nucleotide sequence of ORF3 shows homology of about 48% to *fprA* of *M. xanthus*. The deduced amino acid sequence of ORF3 showed homology of about 43% to FprA, flavin associated protein, from *M. xanthus*. Due to the similarity between ORF3 and the *fprA* gene of *M. xanthus* ORF3 was designated as *fprA*.

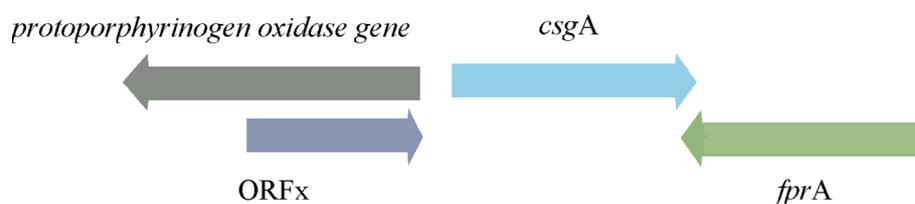


Fig. 2.6. Map of the putative ORFs located on the 3 kbp *XhoI* fragment of *S. aurantiaca*

2.2. Investigation of the physiological function of *CsgA* in vivo; Disruption of the *csgA* gene in *S. aurantiaca*

To analyse the role of CsgA in *S. aurantiaca* development, the *csgA* gene was disrupted by insertional mutagenesis. An internal fragment of the *csgA* gene was replaced by a tetracycline resistance cassette resulting in a CsgA null mutant strain. This *csgA* insertion mutant strain, along with its isogenic parent, was assayed for the ability to form fruiting bodies in response to amino acid starvation.

2.2.1. Construction of plasmid pAM8

In order to inactivate the wild type *csgA* gene in the chromosome of *S. aurantiaca*, plasmid pAM8, harbouring the disrupted *csgA* allele was constructed. Plasmid pAM8 was generated by digesting pAM5 (plasmid that contains the *csgA* locus) with the restriction enzymes *SacI* and *SphI* in order to remove the internal part (340 bp) of the *csgA* gene. After the separation of the restriction mixture by electrophoresis the plasmid fragment of about 6,6 kbp was isolated and purified. Both restriction enzymes generated 5'-overhanging ends that were filled using T4 DNA polymerase to be able to clone the tetracycline resistance cassette into the plasmid. The tetracycline resistance gene from pBR322 was amplified by PCR with the primer pair Tcfw*Xba* and Tcrv*Xba*. Deep Vent Polymerase was used in order to produce blunt ends. Insertion of the tetracycline resistance gene into the 6,6 kbp vector was done according to the standard protocol and *E. coli* strain DH5 α was used for transformation. After selection on LB plates containing tetracycline several colonies were selected, plasmid DNA was purified and used for a restriction analysis. Digestion with the enzymes *XhoI*, or *XbaI*, or *XhoI/SalI* revealed one correct construct. This recombinant plasmid designated pAM8 carried the disrupted *csgA* gene with the tetracycline resistance cassette in the opposite transcriptional orientation of *csgA*. This was verified by sequencing of pAM8 using an internal primer.

2.2.2. Construction of the *csgA* insertion mutant AM8

To construct a *csgA* insertion mutant plasmid pAM8 was linearized with *ScaI* to enhance a double recombination event and transferred into *S. aurantiaca* wild type by electroporation. Tryptone plates containing oxytetracycline were used for selection.

There were two possibilities of integration of the disrupted *csgA* allele from pAM8 into the chromosome of *S. aurantiaca*. The disrupted *csgA* gene may replace the wild type allele by a double recombination event leading to a null CsgA strain. Another possibility is the single homologous crossover between pAM8, carrying disrupted *csgA* allele, and the chromosomal *csgA* locus leading to the integration of the entire plasmid into the genome. Single crossover may occur at the 3' or 5' part of the *csgA* gene resulting in two different merodiploid strains. In both cases two copies of the *csgA* gene are present in tandem separated by the plasmid sequence. One copy of the gene is intact, another one is truncated (Fig 2.7.).

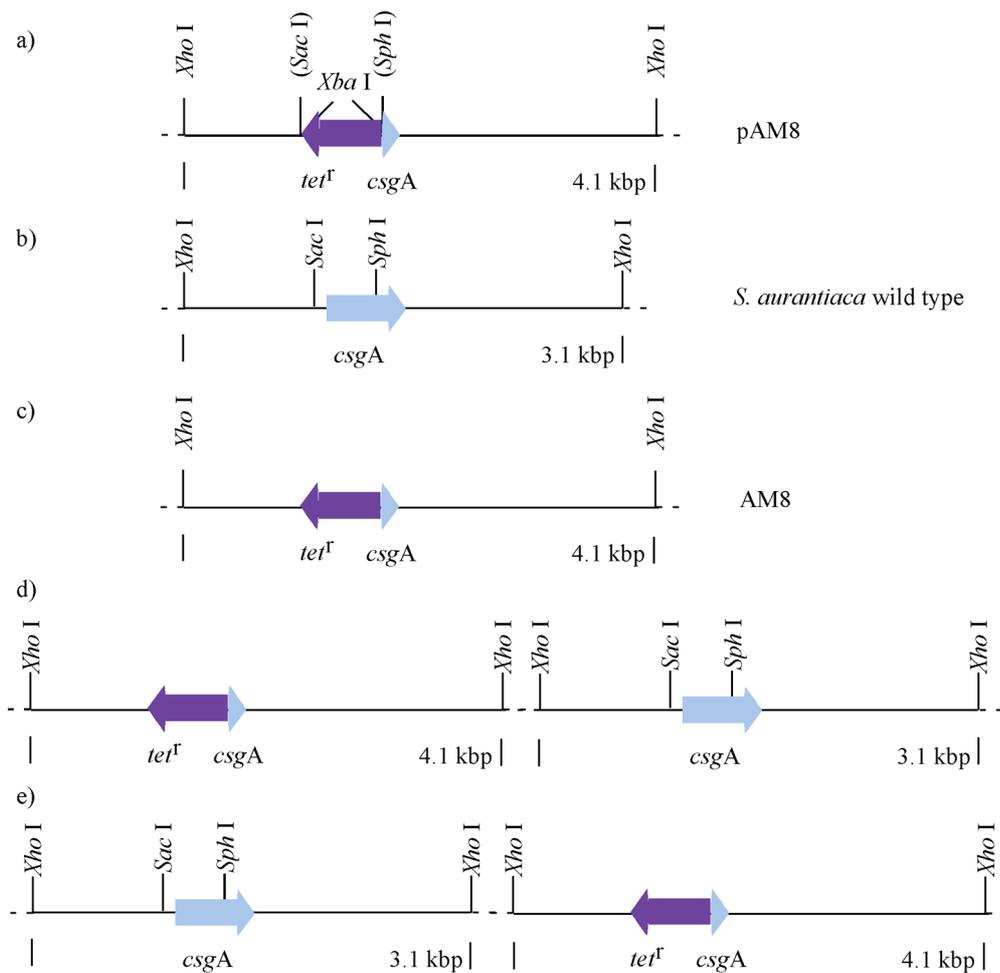


Fig 2.7. Construction of the *csgA* insertion mutant (AM8) a) pAM8 plasmid map; b) *S. aurantiaca* wild type *csgA* locus; c) double recombinant – AM8, *csgA* insertion mutant; d) single recombination event at the 5' end of the gene – merodiploid strain; e) single recombination event at the 3' end of the gene – merodiploid strain.

To distinguish between these two recombination events, Southern hybridisation was performed using different probes. After selection on oxytetracycline six recombinants were obtained and used for further analysis. The biotin labelled plasmid pACYC 177 was used for hybridisation. In the case of a double recombination event no signal should be visible. Chromosomal DNA from the recombinant clones was digested with *Xho*I. Five recombinants showed a positive signal with pACYC 177 leading to the conclusion that all of them were merodiploid mutants with the plasmid pAM8 integrated into the genome. One recombinant clone gave no positive signal with pACYC 177, indicating a double recombination event.

To further investigate the genotype of this putative *csgA* insertional mutant, Southern hybridisation was performed using *Xho*I digested DNA and the biotin labelled tetracycline resistance gene. A 4,1 kbp fragment was detected confirming the integration of the tetracycline resistance cassette into the genome of this recombinant mutant.

Using the 3 kbp *Xho*I fragment (isolated from pAM5, containing the *csgA* gene) as a probe for hybridisation with *Xho*I digested chromosomal DNA of this putative double recombination mutant, a unique 4,1 kbp fragment was detected. This result verified the assumption that *csgA* is indeed inactivated by the tetracycline resistance gene in the strain designated as AM8 (Fig.2.8.).

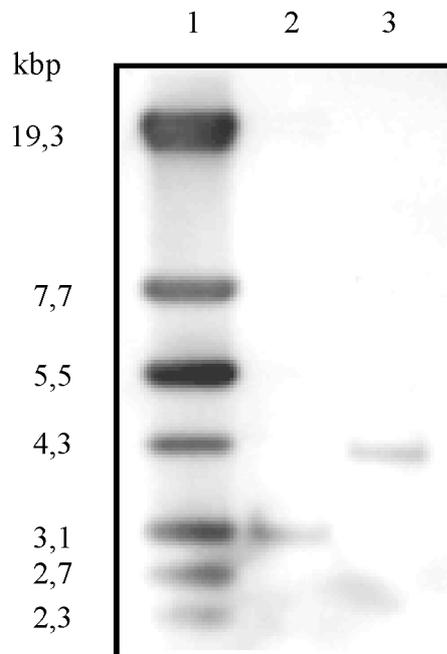


Fig 2.8. Southern hybridisation of *Xho* I digested chromosomal DNA of *S. aurantiaca* wild type and AM8. The 3 kbp *Xho* I fragment from pAM5 was used as a probe.
 Lane 1- DNA Molecular Weight Marker IV (Roche Diagnostics).
 Lane 2- *S. aurantiaca* wild type DNA
 Lane 3- AM8 (*csgA* insertional mutant strain) DNA.

2.2.3. Developmental phenotype of the *csgA* insertion mutant strain

In order to investigate the developmental phenotype of the *csgA* insertion mutant strain fruiting body assay was performed. The same number of the mutant and wild type cells were placed on water agar plates and additionally also on filter papers. Images were taken at different time points after the beginning of starvation (Fig.2.9.).

After 8 hours of development no differences between mutant and wild type cell behaviour were detectable. The spots of mutant and wild type cells had the same size, cell density and the edges of the spots were similar. With progression of the development from 8 to 12 h the first differences between the two strains could be observed. During the indicated time period mutant cells migrated to the outer part of the spot forming a circle with high cell density. Unlike mutant cells, wild type cells concentrated mostly in the inner part of the spot and the edge of the spot was transparent as at the beginning of the development. From 12 to 20 h after the beginning of starvation mutant cells continued to accumulate in an outer circle. They formed aggregation centres very close to each other in the outer ring. Importantly specific rippling trails were not observed with the mutant cells during this time period. Wild type cells formed many aggregation centres between 12 and 20 h after the beginning of development from which fruiting bodies will arise in later stages of the development. Aggregation of the wild type cells was also visible in the inner part of the spot. This is not the case during aggregation of the mutant cells. The rippling that precedes aggregation and overlaps with the early stages of aggregation was well visible when analysing development of wild type cells. After 20 to 26 h mutant as well as wild type cells formed fruiting bodies. No changes in the appearance of the fruiting bodies was observed after 48 h. The mutant fruiting bodies were located in the outer ring of the circle whereas the wild type ones were also present in the inner part of the circle.

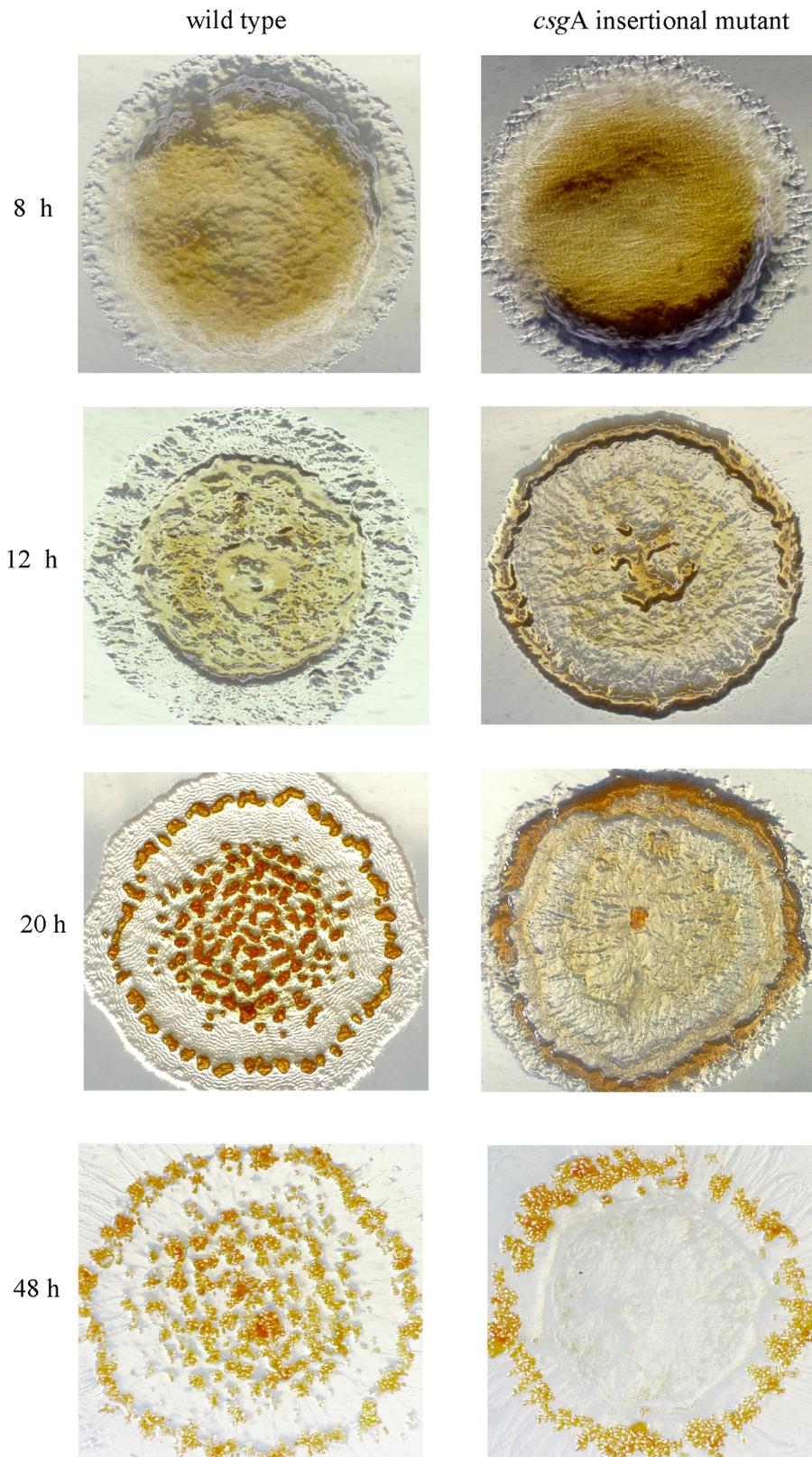


Fig.2.9. Developmental phenotype of *csgA* insertional mutant *versus* wild type. Cells were exposed to starvation on water agar plates for indicated period of time. Spots were viewed from above.

Fruiting body assay performed on filter papers placed on water agar showed even more clear differences in the behaviour of the mutant cells *versus* the wild type ones during development. Wild type cells preferentially stayed in the centre of the circle so that mature wild type fruiting bodies were located in the circle. The mutant cells migrated from the centre of the spot to the outer part during development so that mature fruiting bodies were located more dispersed around the circle (Fig.2.10.).

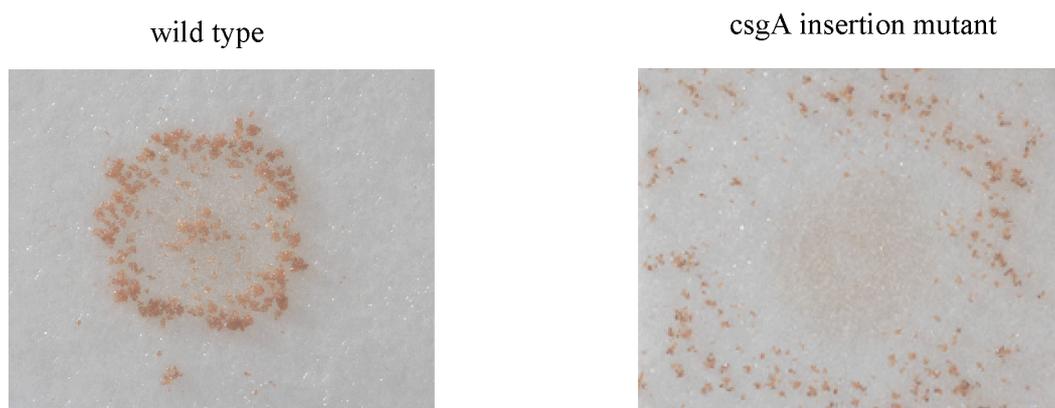


Fig.2.10. Developmental phenotype of *csgA* insertional mutant *versus* wild type. Cells were placed on filter paper located on water agar plates. Spots were viewed from above after 48 h.

The phenotype of the mature mutant fruiting body was the same as the wild type one. Mutant AM8 formed wild type fruiting bodies consisting of a branched stalk bearing several sporangioles (Fig.2.11.).

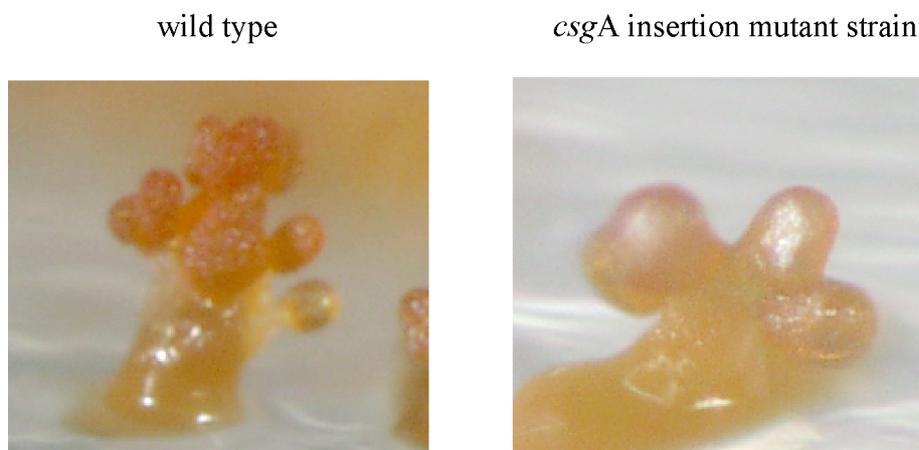


Fig.2.11. Side-view of the representative fruiting body formed by the *csgA* insertional mutant (AM8) and the wild type.

2.2.4. The ability of AM8 myxospores to germinate

The germination assay was performed as described in Materials and Methods. Swarming cells of AM8 or wild type were visible after few days of incubation. This result clearly indicates that myxospores formed by the *csgA* insertional mutant are able to germinate but efficiency of germination is not known.

2.2.5. Ability of the wild type to restore developmental phenotype of mutant AM8

The *csgA* insertional mutant cells do not ripple and show a different migration and aggregation pattern as compared with the wild type cells during development. At that point the question rises if it is possible to restore the developmental phenotype of the mutant by mixing it with the wild type. The experiment was performed by mixing equal amounts of the wild type cells with mutant cells prior to starvation.

Rippling that precedes fruiting body formation was detected in the mixed cell population. Also the aggregation pattern of the mixed population was more or less similar to the wild type when the test was performed on the agar surface. The fruiting body formation testing of the mixed population on filter papers showed different patterns of organisation of the mature fruiting bodies in comparison to the wild type. Wild type as shown previously formed fruits mostly concentrated in the inner part of the circle. The mixed population of the cells formed fruiting bodies concentrated in the circle with additionally some fruits that were dispersed around the circle (Fig.2.12).

Further, rippling waves are detectable in mixed population of the cells and some of the fruits are dispersed around in a mutant like manner.

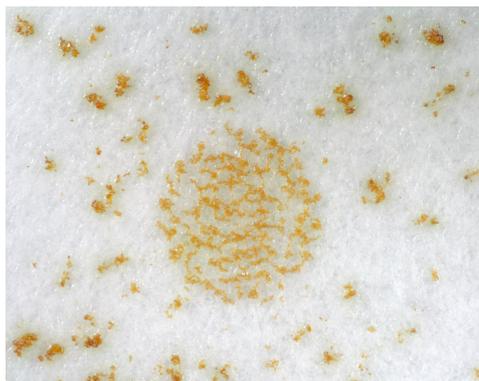


Fig. 2.12. Developmental phenotype of the mixed cells population. Cells were placed on filter paper. Spot viewed from above 48 h after beginning of starvation.

2.2.6. Interaction between WP120 and AM8 mutant cells

Previous results indicated that some fruiting bodies were dispersed around the main circle in the mutant like manner when the mixed population of cells was starved on filter papers. This could be due to the inability of mutant cells to interact with the wild type cells. In this case the fruiting bodies dispersed around would be build up only by the mutant cells. If the mutant cells were able to interact with wild type cells than the fruiting bodies would be build up from a mixture of mutant and wild type cells. This would indicate that the concentration of the signal molecules was very low and not sufficient for all of the cells in the mixed population. The signal molecules could be CsgA *per se* or some product of the CsgA enzymatic activity.

To test this hypothesis WP120 mutant cells were mixed in equal amounts with AM8 and placed on the starvation agar and additionally on the filter papers. The mutant strain WP120 (kindly provided by Wulf Plaga) contains a *gfp* cassette under the control of a strong constitutive *cspA* promoter. The *cspA* gene encodes a small protein belonging to the cold-shock-like protein family. Importantly the mutant cells develop normally and form wild-type fruiting bodies. As a control fruiting body formation was tested with the wild type and WP120 in parallel. After 48 h of development video images of the fruiting bodies were recorded (Fig.2.13.).

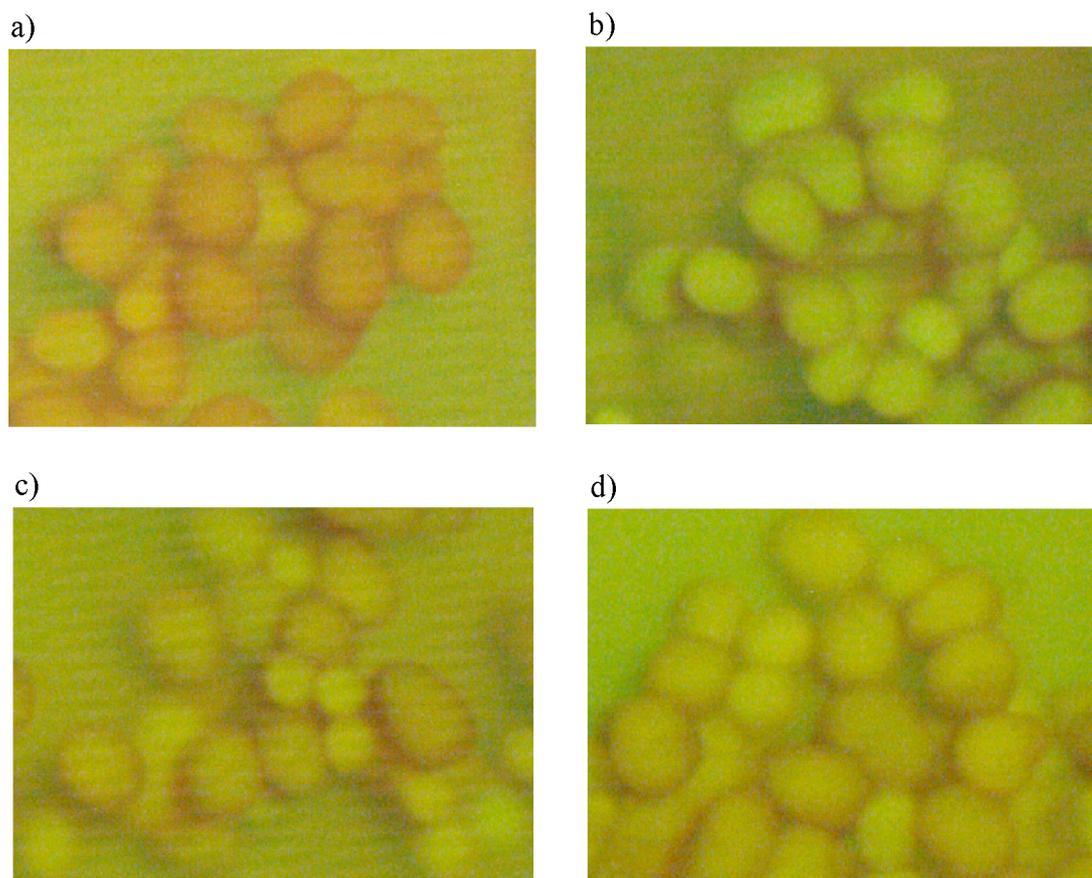


Fig. 2.13. a) Wild type fruiting bodies; b) WP120 fruiting bodies; c) fruiting bodies from the inner part of the circle: mixing experiments of WP120 and AM8 cells; d) fruiting bodies from the ring; mixing experiment of WP120 and AM8 cells.

Mutant WP120 formed in contrast to the wild type (orange colour) green fruiting bodies on starvation medium because it produced green fluorescence protein. The mixture of the AM8 with WP120 formed fruiting bodies of a more intermediate colour. The fruiting bodies from the parts of the ring as well as from the inner part were examined. Clear separation between WP120 and AM8 fruiting bodies were not detectable.

This indicates that mutant cells interact with the wild type cells. The mutant cells have a disrupted *csgA* gene but they retained the ability to respond to CsgA.

2.3. Transcription of *csgA* in *S. aurantiaca*

2.3.1. Determination of the *csgA* expression in the merodiploid mutant AM14

To study the level of the *csgA* gene expression during fruiting body formation, indol induced sporulation or under heat shock conditions, the merodiploid mutant strain AM14 was constructed. The strain contained the wild type *csgA* allele and a *csgA*- \square *trpA-lacZ-neo* fusion allele in tandem. The increase of \square -galactosidase activity in the merodiploid mutant is an indication of the transcriptional level of *csgA* in *S. aurantiaca*.

2.3.1.1. Construction of plasmid pAM14

In order to produce a *csgA* merodiploid mutant strain, a plasmid harbouring the functional *csgA* promoter fused to a reporter gene was constructed and integrated into the *S. aurantiaca* wild type *csgA* locus *via* homologous recombination. As reporter the promoterless \square *trpA-lacZ* gene from the plasmid Tn5 *lacZ*1 was used. It contains the whole *lacZ* gene and about 150 bp of the *trpA* gene fused to the 5' end of *lacZ*. The *trpA* gene has stop codons in all three reading frames to prevent a translational fusion.

A 0,9 kbp fragment harbouring 0,6 kbp of the *csgA* promoter, upstream and 0,3 kbp downstream of putative ATG start codon, was amplified by PCR with the primer pair *NotI csgA* 21 and *csgA* 20 *XbaI* using the previously described plasmid pAM5 (plasmid that contains the *csgA* locus) as a template. The primers contained additional restriction sites for *NotI* and *XbaI* for directional cloning of the PCR product in plasmid pSM62 (kindly provided by S. Müller). Plasmid pSM62 (derivative of pBSSK-) harbours the promoterless \square *trpAlacZ* gene fused to a *neo* cassette for selection of recombinants after transformation. Insertion of the *csgA* promoter sequences to \square *trpAlacZ-neo* gene led to the plasmid pAM14. The plasmid sequence was reconfirmed by restriction analysis and sequencing.

2.3.1.2. Constructions of the merodiploid mutant strain AM14

Plasmid pAM14 was integrated into the *S. aurantiaca* chromosome *via* a single recombinational event leading to a merodiploid mutant strain (AM14) with the wild type *csgA* allele and a *csgA* promoter- Δ *trpA**lacZ*-*neo* fusion allele in tandem (Fig. 2.14.).

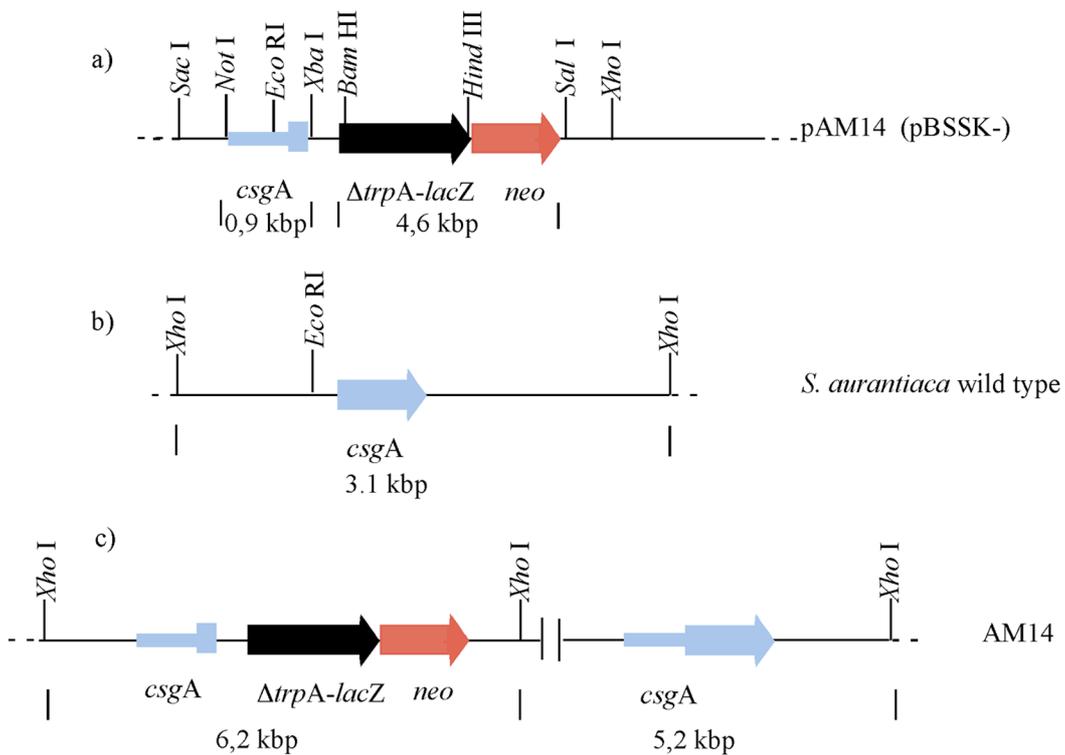


Fig. 2.14. Construction of the merodiploid strain AM14; a) pAM14 plasmid map; b) *S. aurantiaca* wild type *csgA* locus; c) single recombination - *csgA*- Δ *trpA-lacZ*-*neo* fusion strain (AM14).

After electroporation recombinants were selected on tryptone plates containing kanamycin sulfate. Several colonies were obtained and analysed further.

To prove the correct integration of the plasmid pAM14 into the *csgA* locus, chromosomal DNA isolated from the recombinants was restricted with *Xho*I and used in a Southern blot analysis with the biotin labelled *neo* gene as a probe. In case of a correct

plasmid integration into the *csgA* chromosomal locus restriction of the DNA of the recombinants DNA with *Xho*I should lead to one band of about 6,2 kbp that hybridises with the probe. One merodiploid mutant strain, AM14, was shown to have the correct genotype as shown by Southern blot analysis (Fig.2.15.).

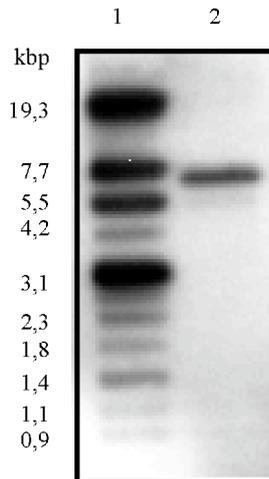


Fig.2.15. Southern hybridisation of *Xho* I digested chromosomal DNA of AM14 with *neo* gene as a probe.

Lane 1- DNA Molecular Weight Marker IV (Roche Diagnostics).

Lane 2- AM14 merodiploid mutant strain DNA

2.3.1.3. Determination of the β -galactosidase activity in AM14

For analysing *csgA* expression during development, fruiting body assays were performed with the merodiploid mutant strain AM14 and the wild type. Cells were scraped off the water agar at different time points from the beginning of starvation. Cell extracts were assayed for a β -galactosidase activity with the fluorescence substrate 4-MUG. As control the protein extracts isolated from wild type cells were used in the assay in order to evaluate the background signal.

No significant β -galactosidase activity was detected in the vegetative state of the merodiploid mutant in the fluorometric assay (10 μ g of the total protein extract was used). Only a very low increase of the β -galactosidase activity was detected about 8 h after the beginning of starvation (Fig.2.16). No significant β -galactosidase activity was detected during indol induced sporulation or under heat shock conditions.

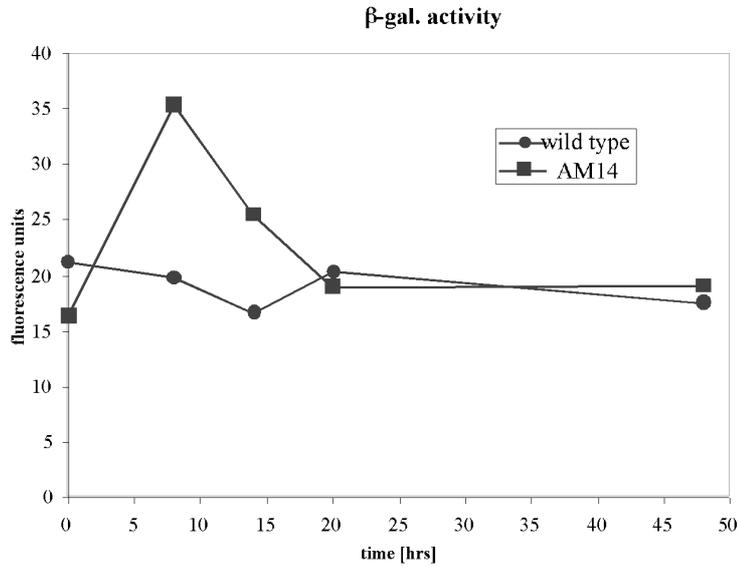


Fig. 2.16. Determination of the β -galactosidase activity of strain AM14 during fruiting body formation (10 μ g). The level of the β -galactosidase activity is indicated as black squares. The level of the background signal is indicated as black dots.

The fluorometric assay was repeated under the same experimental conditions but with five times more total protein. β -galactosidase activity was detected during development under this experimental condition (Fig.2.17). The merodiploid mutant (vegetative state) showed a very low level of β -galactosidase activity. The activity slightly increased with the progression of starvation. The maximum activity was detected about 8 h after the beginning of development. The level of the background signal was estimated by a control experiment with 50 μ g of total protein isolated from wild type cells at the same time points after initiation of starvation. A low level of the β -galactosidase activity was also detected 30 min after indol or heat shock treatment.

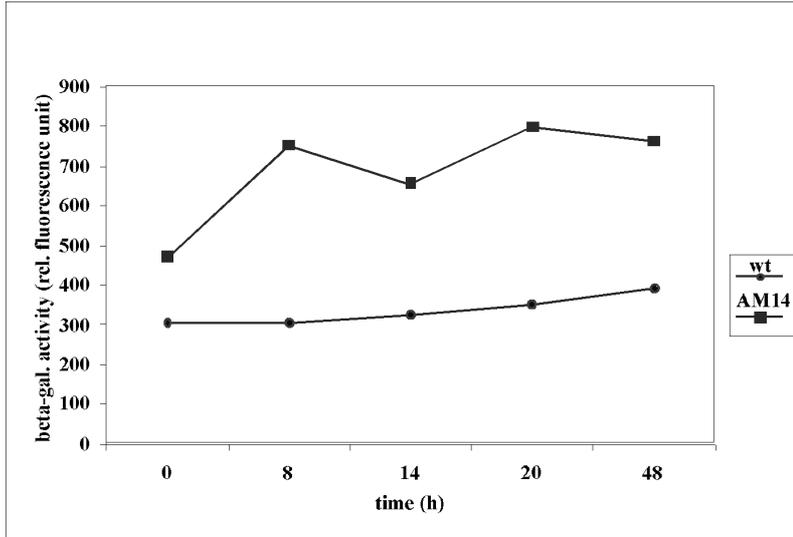


Fig. 2.17. Determination of the β -galactosidase activity of strain AM14 during fruiting body formation (50 μ g). The level of the β -galactosidase activity is indicated as black squares. The level of the background signal is indicated as black dots.

2.3.1.4. Detection of the β -galactosidase activity *in situ*

The merodiploid mutant was placed on starvation agar supplemented with 25 μ g of X-gal per ml. Starvation of the merodiploid mutant under these conditions resulted in fruiting bodies whose stems were stained in blue in the course of about 3 days after the beginning of development (Fig.2.18.).



Fig.2.18. Fruiting body of the merodiploid strain AM14 on starvation agar containing X-gal.

2.3.1.5. Developmental phenotype of AM14

To examine the phenotype of the merodiploid mutant strain AM14 containing the wild type *csgA* allele with 0,6 kbp of the upstream sequence from the putative ATG start codon, fruiting body assay was performed. The same number of mutant and wild type cells were exposed to starvation on water agar and filter papers. The fruiting assay showed no difference in the developmental behaviour of the merodiploid mutant cells AM14 as compared to wild type cells. During the first hours of starvation the mutant cells showed no difference in the migration pattern. The mutant cells preferentially stayed in the inner part of the circle as it is also observed for the wild type. After 20 h of starvation, AM14 cells had formed aggregation centres and the cells were rippling in the same way as well as wild type cells. The aggregation pattern of the mutant was also similar to that of wild type cells. Aggregation centres of AM14 cells formed into normal fruiting bodies containing a stalk with several sporangioles in the same time course as observed for the wild type (Fig.2.19.).

Spores formed by the merodiploid strain AM14 were able to germinate again after incubation under suitable nutrient conditions comparable to wild type spores.

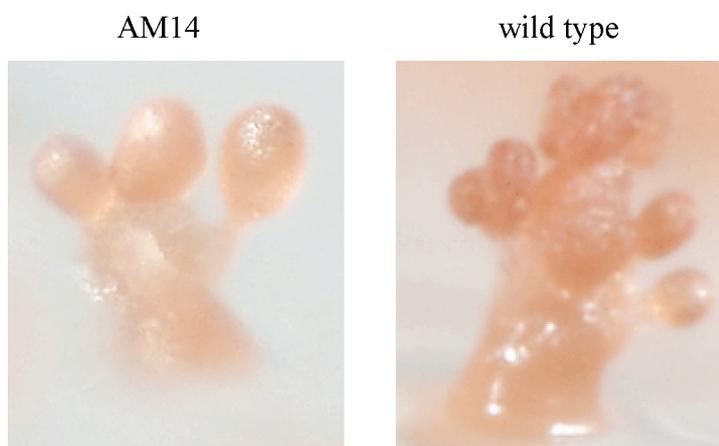


Fig. 2.19. Side-view of representative fruiting bodies formed by AM14 and wild type after 48h of starvation on water agar.

Mutant AM14 displayed the same behaviour during development and formed wild type fruiting bodies enclosing viable spores. This led to the conclusion that the upstream region of 0,6 kbp used to construct the merodiploid mutant is sufficient for expression of the *csgA* gene.

2.3.2. Detection of *csgA* expression by RT-PCR

Additionally the transcription of the *csgA* gene in *S. aurantiaca* during vegetative growth, development, or artificially induced sporulation was determined by qualitative RT-PCR. Total RNA isolated from cells under different conditions was transcribed with AMV reverse transcriptase and cDNA was amplified with primer pair *csgA7/csgA8* as described in the method part. The primers hybridize downstream from the GTG start.

The transcription of *csgA* was detected during vegetative growth as well as during development (Fig.2.20). The *csgA* expression was detected after 8, 20, 30 h of development. Additionally expression was also detected during artificially induced sporulation with indol after 10, 30, 60 and 120 min.

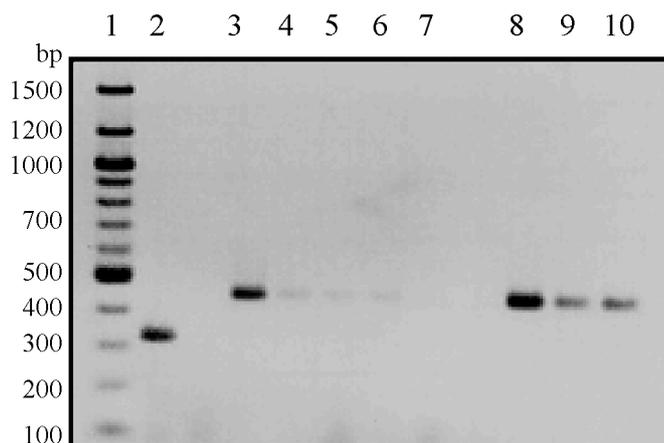


Fig.2.20. Expression of *csgA* detected by RT-PCR. The RT-PCR reactions were carried out using the same amounts of total RNA. Lane 1: 100 bp DNA Ladder (New England BioLabs); lane 2: control provided by kit (Promega); lane 3: vegetative cells; lane 4: indol induced sporulation after 10 min; lane 5: indol induced sporulation 30 min; lane 6: indol induced sporulation 60 min; lane 7: indol induced sporulation 120 min, lane 8: cells after 8h of development; lane 9: cells after 20h of development, lane 10: cells after 30h of development.

2.4. Production of CsgA in *S. aurantiaca*

For immunological identification of the *csgA* encoded protein during *S. aurantiaca* growth and development, polyclonal antisera were raised against a fusion protein containing a specific part of CsgA and two CsgA peptide sequences.

2.4.1. Heterologous expression of the fragment encoding antigenic determinants of CsgA

A recombinant protein containing part of CsgA fused to 6xHis-DHFR was purified and used for immunisation of two rabbits. The production of the CsgA protein in *S. aurantiaca* was monitored by Western blot analysis with polyclonal antisera.

2.4.1.1. Cloning the DNA fragment encoding the CsgA antigens into the pQE42 expression vector

The central part of the *csgA* gene (465 bp) encoding a putative polypeptide of 155 amino acids was chosen for the immunisation of rabbits as this region shows a high antigenicity index (Fig.2.22.a).

The 465 bp *csgA* fragment was generated by PCR using chromosomal DNA from *S. aurantiaca* wild type as a template with the primer pair *Bgl*III *csgA*7 and *csgA*8. The obtained PCR product was digested with *Bgl*III and cloned into the polylinker site of the pQE42 vector (Qiagen) predigested with *Bgl*III/*Sma*I. Upstream of the pQE42 polylinker site there are six histidine codons and a gene that encodes the mice dehydrofolate reductase (DHFR). The integration of the *csgA* sequence in frame into the polylinker leads to a hybrid gene that encodes a fusion protein designated 6xHis-DHFR-CsgA, respectively. The expression of the fusion protein was under the control of the T5 promoter fused to the *lac* operator sequence. Parts of the ligation reaction were used for the transformation of *E. coli* M15(pREP4). The helper plasmid pREP4 encodes the *lac* repressor which binds to the *lac* operator sequence and represses transcription initiated at the T5 promoter. The addition of IPTG induces expression of the recombinant protein since IPTG binds to the *lac* repressor protein and inactivates it.

Various transformants were screened by preparing small scale expression cultures. Protein extracts were separated on a 12,5% SDS polyacrylamide gel. The 6xHis-DHFR-CsgA fusion protein was expected to migrate at about 43 kDa on the SDS gel since DHFR migrates at about 26 kDa and the calculated molecular weight of the CsgA fragment is about 17 kDa. Protein extracts isolated from some transformants contained

one protein at about 43 kDa matching the size of the fusion protein. The identity of the fusion protein was verified by western analysis using anti-DHFR-antibody (Fig.2.21.).

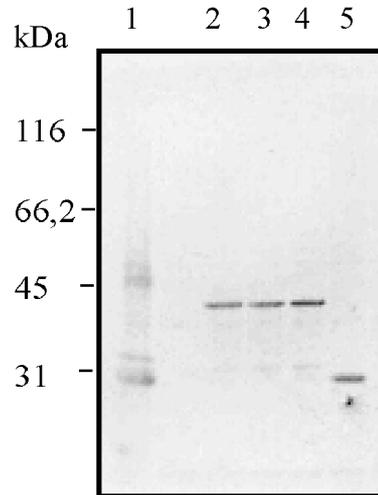


Fig.2.21. Immunoblot analysis of protein extracts isolated from four different transformants reacted with anti-DHFR antibodies. The protein sizes were estimated with a broad range protein marker (BioRad). Lane 1-purified DHFR –26 kDa; lane 2-4 total proteins isolated from three positive transformants; lane 5-protein isolated from a transformant containing religated plasmid pQE42.

From positive transformants, plasmids were isolated and sequenced to verify the fusion construct. Plasmid pAM3 contains the internal part of the *csgA* gene cloned in frame into the expression vector pQE42 (Fig.2.22.b).

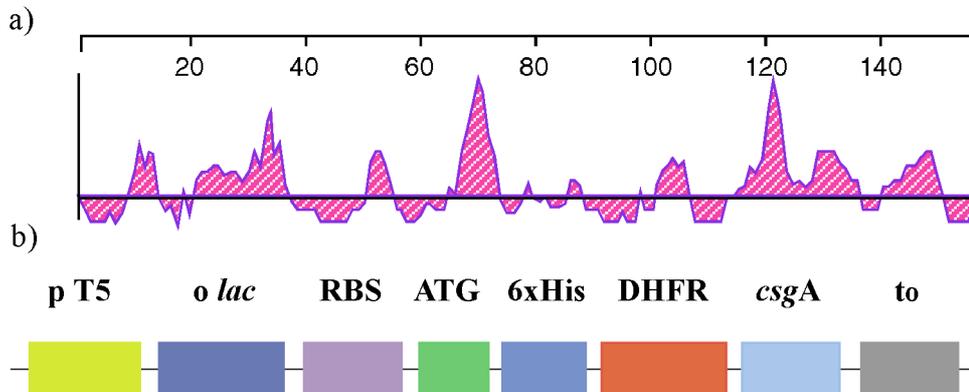


Fig.2.22. a) Antigenic index (Jameson-Wolf) of 155 amino acids encoded by *csgA* calculated by using the program Protean from the Macintosh Lasergene package.

b) part of the pAM3 plasmid.

Heterologous expression of CsgA in the *E. coli* strain M15(pREP4) resulted in a very low concentration of the fusion protein. In order to increase the level of recombinant protein, pAM3 was transferred into the *E. coli* strain SG13009 (Qiagen). No significant increase in the concentration of the recombinant protein was detected after induction with IPTG.

2.4.1.2. Purification of the recombinant protein, 6xHis-DHFR-CsgA, under denaturing conditions

The fusion protein was partially purified from M15 (pREP4) *E. coli* cells containing the plasmid pAM3 by FPLC on a Ni-NTA column under denaturing conditions (6 M GuHCl and 8 M Urea). Elution of the 6xHis-DHFR-CsgA from the column was achieved with 200 mM imidazol. The eluted fraction contained the fusion protein as the major component and also some proteins from *E. coli* that bind to the Ni-NTA column. The fusion protein was further purified by preparative SDS-PAGE. Recombinant protein eluted from the Ni-NTA column was visualised by staining the SDS gel with Coomassie blue and its identity was confirmed by Western blot analysis using an anti-DHFR antiserum (Fig.2.23).

After purification on a Ni-NTA column protein fractions were concentrated by extraction of the solvent with Sephadex 50 and further purified on preparative SDS-PAGE. The gel slice containing the recombinant protein was cut out from the gel and was used directly without removing the DHRF part to produce antisera in rabbits.

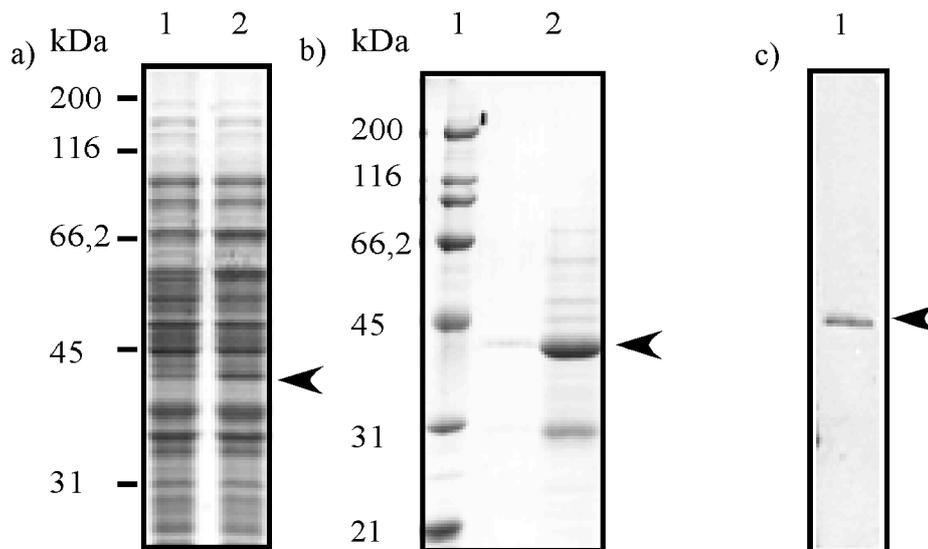


Fig.2.23.a) Synthesis of 6xHis-DHFR-CsgA in *E. coli* M15 (pREP4). Fractions were visualized by Coomassie blue staining after separation on a 12,5% SDS PAGE. 1-lane cell lysates of a noninduced control; 2-lane lysates of IPTG (1mM) induced cells. The arrow indicates the predicted recombinant protein band. The protein sizes were estimated with a broad range protein marker (BioRad).

b) Purification of 6xHis-DHFR-CsgA on a Ni-NTA column. The polypeptides of the fraction were visualized by Coomassie blue staining after separation on a 12,5% SDS PAGE gel. 1-lane broad range protein marker (BioRad); 2-lane protein fraction after purification on a Ni-NTA column. The arrow indicates the predicted recombinant protein band

c) Verification of the 6xHis-DHFR-CsgA in purified protein fraction by Western blot analysis with anti-DHFR-antibodies. 1-lane protein fraction eluted from the Ni-NTA column. The arrow indicates the predicted recombinant protein band.

2.4.1.3. Production of CsgA in *S aurantiaca*

In order to determine the production of CsgA in *S. aurantiaca* immunoblot analysis was performed with total protein extracts using anti-6xHis-DHFR-CsgA antibodies. Proteins were isolated from the wild type or a *csgA* insertion mutant cells after different periods of development.

Fig.2.24. shows the western analysis of proteins from the wild type and the *csgA* mutant isolated from vegetative cells and after 16 h of development. The CsgA protein was not detectable in total protein extracts from the wild type (vegetative state) and during development (16 h). One cross-reacting polypeptide with apparent molecular mass of about 14 kDa was detected in the protein extracts from the wild type and the

csgA mutant. A cross-reacting polypeptide of about 14 kDa was detected also in wild type and *csgA* mutant cells in western blot analysis using preimmune sera.

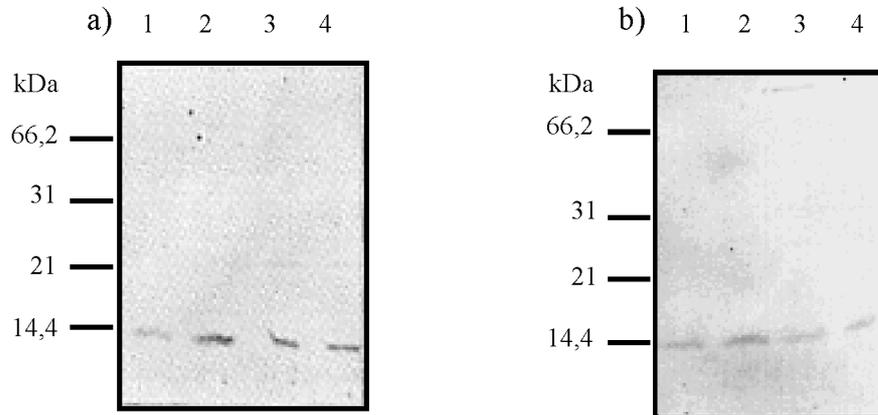


Fig.2.24. Immunoblot analysis of total protein extracts from the wild type and the *csgA* knock out mutant **a)** anti-6xHis-DHFR-CsgA-antibody and **b)** preimmune serum. Total protein added per lane was 10 μ g (corresponding to about 1×10^8 cells). Protein migration was estimated with a broad range protein marker (BioRad). The proteins were electroblotted onto a PVDF membrane. The goat anti-rabbit antiserum conjugated with alkaline phosphatase was used as the second antibody.

Lane 1 and 2- total protein from wild type (vegetative state) and 16 h after the beginning of development. Lane 3 and 4- total protein from *csgA* mutant (vegetative state) and 16 h after the beginning of development.

Bands that correspond to the calculated molecular weight of the three possible CsgA polypeptides of a various sizes were not detected in wild type cells, using the anti-6xHis-DHFR-CsgA antibodies, even with more sensitive detection method (ECL) (Fig.2.25.). Similar proteins from the wild type or the *csgA* insertional mutant cells (vegetative state or after 20 h of development) cross-reacted with these antibodies. The same cross-reacting polypeptides were detected in the protein extracts from the wild type and *csgA* mutant cells after 30 min induction with indol. The sensitivity of the antiserum was tested with the fusion protein (6xHis-DHFR-CsgA). The 10 μ g of the fusion protein was not detected by the antiserum. Additionally total protein extract from *M. xanthus* cells in the lane 4 (Fig.2.25.) was used as a control in the western analysis using *S. aurantiaca* anti-6xHis-DHFR-CsgA serum. Predicted protein bands corresponding to *M. xanthus* CsgA (24 and 17 kDa) were not detectable.

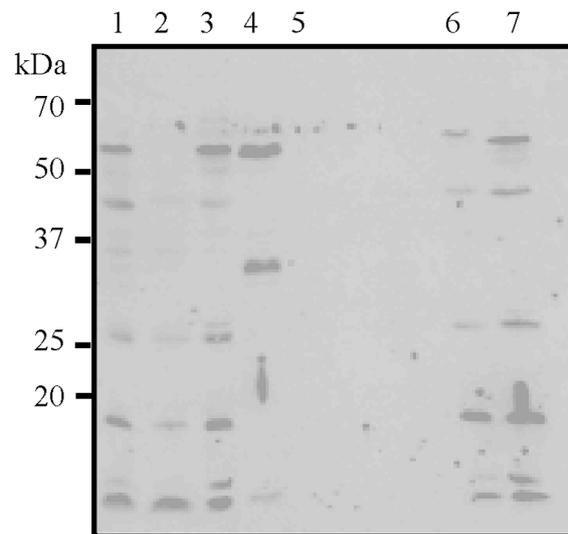


Fig 2.25. Immunoblot analysis of total proteins from *S. aurantiaca* wild type or *csgA* knock out mutant cells and *M. xanthus* vegetative cells reacted with anti-6xHis-CsgA-antibody. Total proteins added per line were 10 μ g (correspond to about 1×10^8 cells). The proteins were electroblotted onto a PVDF membrane. The peroxidase-cojugated goat anti-rabbit secondary antibody was used. Protein migration was estimated with a prestained protein marker (BioRad); lanes 1 and 2: total protein isolated from wild type cells vegetative state or 20 h after the beginning of starvation; lane 3- protein isolated from wild type cells 30 min after addition of indol; lane 4- protein isolated from vegetative *M. xanthus* cells; lane 5- fusion protein 6xHis-DHFR-CsgA 10 μ g; lane 6- protein isolated from *csgA* mutant cells 20 h after beginning of development; lane 7- protein isolated from *csgA* mutant cells 30 min after addition of indol.

2.4.1.5. Production of anti-peptide antibodies

Two peptides were synthesised in order to produce anti-peptide antibodies against the CsgA protein. The generated peptides were termed CsgA-1 and CsgA-2, respectively. Peptides were both 21 amino acids long and included a cystein residue at the C terminus. The cystein residue was added as an extra amino acid to allow simple, one site coupling *via* the free sulfhydryl group to the carrier keyhole limpet hemacyanin (KLH). Peptides were coupled to the carrier protein in order to be exposed at the surface of the protein and therefore to be recognised as epitopes from the animal immune system.

The peptide sequences displayed a high antigenic (Jameson-Wolf) index and high hydrophilicity (Kyte and Doolittle, 1982) index (Fig.2.26.). A proline residue was

enclosed in both peptides since the presence of proline residues in synthetic peptides originally was suggested. It was suggested that peptides containing hydrophilic amino acids and proline residues were more likely to be exposed on the surface of the native protein than other sequences (Kyte and Doolittle, 1982). Hydrophilic peptides are more soluble and thus can be coupled more easily.

The mixture containing equal amounts of CsgA-1 and CsgA-2 peptides were used for the immunisation of two different rabbits.

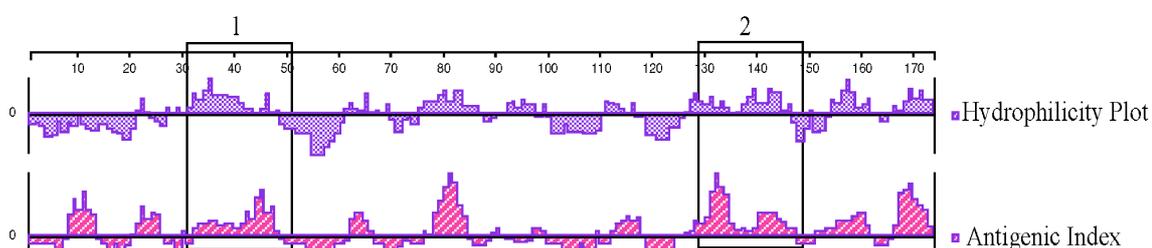


Fig. 2.26. Antigenic index and hydrophilicity plot of the CsgA -pep1 (box 1) and CsgA-pep2 (box 2). Amino acid sequences of a CsgA-1: MDYEDMTKVMETNSVGPMRLC and a CsgA-2: VRTEMGGKLAPMRPEDAVRG.

Anti-peptide antibodies were used in a western blot analysis of total protein isolated from the wild type and the *csgA* mutant cells. The cells were scraped from starvation agar after different time points after the beginning of development and total protein was isolated.

Total protein from the wild type and the *csgA* mutant isolated from vegetative cells and after 20 h of development. These protein preparations were used for Western blot analysis reacted with the antiserum raised against the mixture of the peptides CsgA-1 and CsgA-2. Protein extracts from wild type and *csgA* mutant cells contained mainly three cross-reacting polypeptides with apparent molecular masses of about 70 kDa, 50 kDa and 10 kDa. Cross-reacting polypeptides, that have a calculated molecular mass of CsgA protein, were not detected in the wild type cells (Fig.2.27).

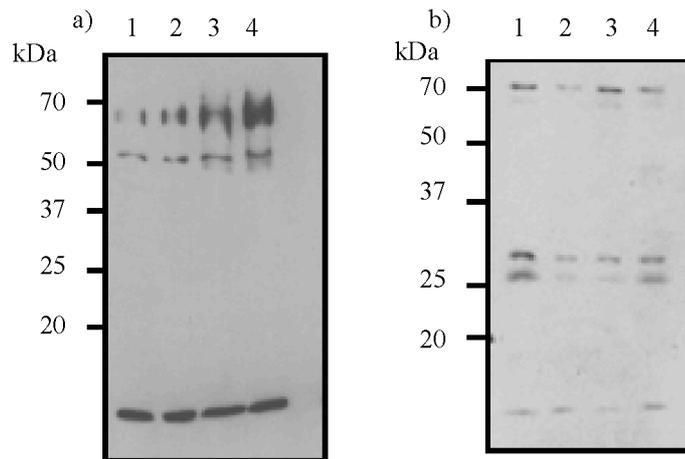


Fig.2.27. a) and b) Immunoblot analysis of total protein extracts from wild type and *csgA* knock-out mutant. **a)** anti-peptide-sera and **b)** preimmune sera. 10 μ g of total protein added per lane. The size of the polypeptides was estimated with the prestained protein marker (BioRad). Lane 1 and 2- total protein from vegetative wild type and 20 h after the beginning of development. Lane 3 and 4- total protein from vegetative *csgA* mutant cells and 20 h after the beginning of development.

As control the western blot was performed with the total protein extracts isolated from the wild type and the *csgA* mutant cells, vegetative state and after 20 h of development, using the preimmune sera. Four cross-reacting polypeptides with an apparent molecular mass of about 70 kDa, 30 kDa, 25 kDa and 10 kDa were detected (Fig.2.27.).

In order to avoid cross-reactions with the polyclonal serum affinity purified anti-peptide antibodies were used. The serum was purified on affinity columns, which enclosed one of the peptide, CsgA-1 or CsgA-2. Purified anti-CsgA1 antibodies and anti-CsgA2 antibodies were used in western blot analysis of the total protein from wild type and *csgA* mutant cells, isolated after different time periods from the beginning of starvation, 30 min after the indol induced sporulation or 30 min after the heat-shock treatment. Only one cross-reacting protein with an apparent molecular mass of about 80 kDa was detected with the anti-CsgA-1-antibodies in total proteins extracts of wild type and mutant cells (Fig.2.28.a) There is little if any reaction with the anti-CsgA-2-antibodies indicating that the antibodies are specific (Fig.2.28.b).

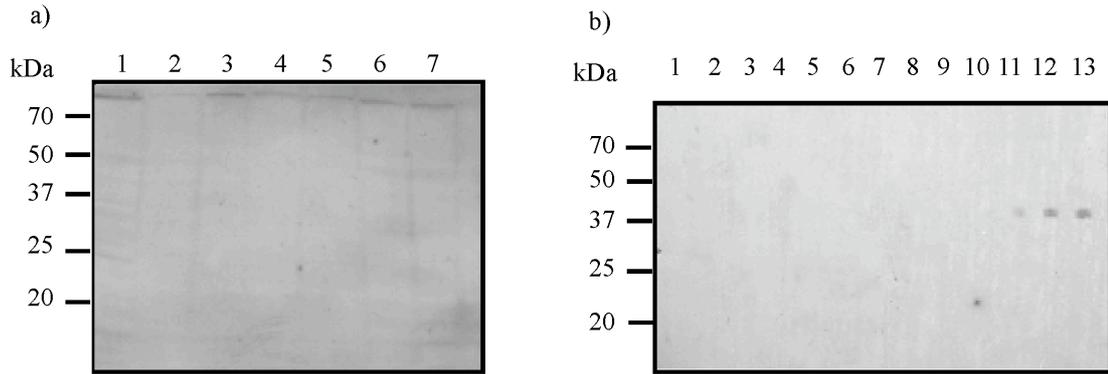


Fig.2.28. Immunoblot analysis of total protein isolated from wild type and *csgA* mutant cells reacted with **a)** anti-CsgA-1- antibody. 100 μ g of protein was added per each lane. Protein migration was estimated with the prestained protein marker (BioRad). Total protein isolated from lane 1: vegetative wild type cells; lane 2: wild type cells after 8 h of development; lane 3: wild type cells at 14 h of development; lane 4: wild type cells at 20 h of development; lane 5: wild type cells 30 min after heat shock at 37 °C, lane 6: wild type cells 30 min after addition of indol; lane 7: vegetative *csgA* mutant cells.

b) anti-CsgA-2- antibody. Lane 1: total protein isolated from lane vegetative wild type cells; lane 2, 3 and 4: extracts of wild type cells after 8, 14 and 20 h of development, respectively; lanes 5 and 6: protein extracts of wild type cells after heat shock (30 min 37°C) and after indole treatment for 30 min, respectively; lane 7: vegetative *csgA* mutant cells; lane 8: *csgA* mutant cells at 20 h of development, lanes 9-13: fusion protein (6xHis-DHFR-CsgA) 1ng; 2 ng; 3ng; 5 ng and 10 ng.

The CsgA protein was not detected in protein extracts of vegetative cells and after different time of development, under these experimental conditions.

The CsgA is expected to be a minor fraction of *S. aurantiaca* total proteins. Fig.2.29. shows total proteins of *S. aurantiaca* cells isolated after different time periods of development.

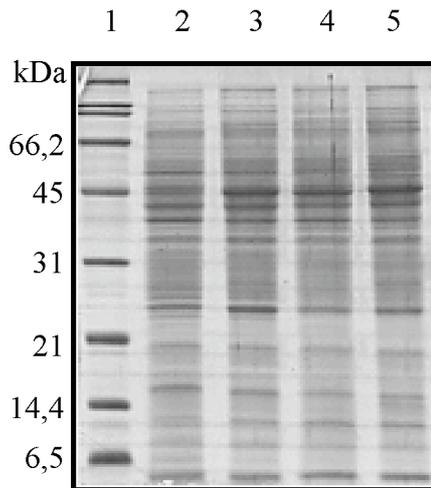


Fig.2.29. lane 1- broad range protein marker IV, lanes 2-5- 10 μ g of total protein isolated from wild type cells: vegetative, 8 h, 14 h and 20 h after the beginning of development, respectively.

Additionally, in order to test the representation of the isolated protein extracts from *S. aurantiaca*, immunoblot analysis was performed with anti-CspA-antibodies (kindly provided by Wulf Plaga). The serum recognised a protein of the expected size of 5,5 kDa in total protein extracts isolated from wild type and *csgA* cells (Fig.2.30.).

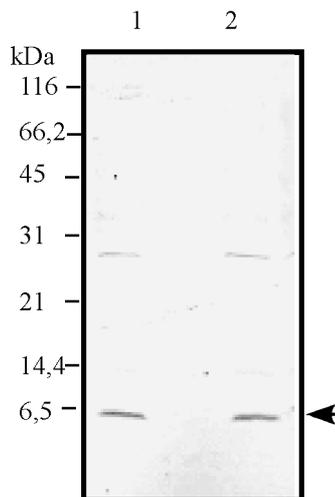


Fig. 2.30. Production of CspA in *S. aurantiaca* wild type (lane 1) and *csgA* mutant (lane 2) during vegetative growth. The sizes of a protein were estimated with a broad range protein marker (BioRad).

III. Discussion

S. aurantiaca is a social living bacterium, highly communicative, and represents therefore an excellent model organism to study intercellular signalling in prokaryotes. Its complex life cycle strongly depends on different signals exchanged between the cells during development as well as during vegetative growth.

Identification of these signal molecules and their corresponding signalling pathways would contribute to the understanding of the complex life cycle of these bacteria. Thus, it would also give some more answers to the question how genes can control development. Differential gene expression in the time course of development is tightly regulated by a sophisticated communication network in these bacteria.

In contrast to the other well-studied myxobacterium *M. xanthus* research on intercellular signalling in *S. aurantiaca* is just at the beginning. Additionally for *S. aurantiaca* less bacterial genetic methods are available for manipulation (Schairer, 1993). Stigmolone, a pheromone, is the only isolated and characterized signal molecule of *S. aurantiaca*. In addition, several genes were identified whose products might have a role in cell-cell communication.

One of the previously identified genes, homolog to the *M. xanthus* *csgA*, was further characterized in this work. The function of the *S. aurantiaca* CsgA protein in intercellular communication was elucidated by inactivation of its encoding gene. The detectable phenotype that was observed as a consequence of *csgA* inactivation by insertional mutagenesis suggests an involvement of CsgA in intercellular communication of *S. aurantiaca*.

3.1. *S. aurantiaca* *csgA* gene locus

In *S. aurantiaca* the *csgA* locus was localized on a 3 kbp *XhoI* DNA fragment. It contains the *csgA* gene and three putative ORFs localized upstream and downstream of the gene.

A detailed sequence analysis of the *csgA* upstream region revealed two additional putative start codons (ATG). They are located in frame upstream of the previously

proposed GTG start of the gene. It was suggested that *csgA* starts with a GTG codon, without Shine-Dalgarno sequence, and ends with a TAG stop codon. Thus, the proposed gene encodes a protein of 173 amino acids with the predicted molecular mass of about 19 kDa (Butterfass, 1992).

Alignment studies with the *M. xanthus csgA* gene revealed the best matching when the *S. aurantiaca csgA* gene starts with an ATG codon located 189 bp upstream of the GTG codon. If this ATG is used as a real translational start, CsgA would be 236 amino acids long with a calculated molecular mass of about 26 kDa. No Shine-Dalgarno sequence could be identified in front of the ATG start codon. Consensus translation sequences in *S. aurantiaca* were not determined up to now because just a small number of genes are identified. Additionally most of the genes start with an ATG codon (*hspA*, *sigA*, *mtaA*, *mtaB*, *fbfA*, *fbfB*, *fbfC*) (Heidelbach et al., 1993, Müller, 2002; Silakowski et al., 1996; Silakowski et al., 1998; Silakowski et al., 1999; Skladny et al., 1994).

The C+G content at the third position of codons used in the *csgA* gene is high (about 89%) which agrees well with the codon usage in myxobacteria. The C+G content in the second position is 46% and 65% at the first position. The myxobacteria have a genome with extremely high C+G content of 62-72% (Chen et al, 1990). The genes from an organism with a C+G content greater than 54 % have a pattern in which the C+G content of the third codon position is greater than the C+G content of the first position, which is in turn greater than C+G content of the second position (Bibb, et al., 1984). The C+G content of myxobacteria is significantly higher than that of the *E. coli* genome 50,5 %. Therefore standard rules for finding Shine-Dalgarno sequences optimised for the *E. coli* cannot be applied for myxobacteria.

Another ATG codon is located 348 bp upstream from the first reported GTG start. It is preceded by a Shine-Dalgarno sequence but in an unfavourable context (only 2 bp from ATG). If this codon is used as a translational start site, the *csgA* gene would encode a protein of 289 amino acids with a calculated molecular mass of about 32 kDa.

These others possible start codons cannot be excluded as putative translational starts of the *csgA* coding region. They could also serve as second initiation site for the *csgA*

encoded protein. To investigate the real initiation site of *csgA* it is necessary to perform further experiments. Insertional mutagenesis in the upstream region of the putative start codons or site-directed mutagenesis of all three putative start codons are necessary for the determination of the real translation start of *csgA* in *S. aurantiaca*.

Upstream of the *csgA* gene an ORF that is arranged in a divergent orientation was identified. The gene has a homology of about 60 % to the protoporphyrinogen oxidase gene of *M. xanthus*. Protoporphyrinogen oxidase catalyses the six electron oxidation of protoporphyrinogen IX to protoporphyrin IX during the heme biosynthesis pathway (Dailey and Dailey 1996). The enzyme is oxygen dependent and contains flavin as a cofactor. The gene product is not involved in development. Purified *M. xanthus* protoporphyrinogen oxidase shows similarity to the mammalian enzyme and the enzyme from *B. subtilis*. The function of the putative gene product of *S. aurantiaca* remains to be investigated.

The other putative ORF identified upstream of the *csgA* gene did not show homology to known proteins up to now.

Another putative gene was identified downstream of *csgA* arranged in a divergent orientation, that shows homology of about 48% to the *fprA* gene from *M. xanthus*. The *fprA* gene encodes a flavin associated protein A with a vital function in *M. xanthus* (Shimkets, 1990). Efforts to replace the wild type allele with a mutant allele was not successful. This suggests that *fprA* is important for vegetative growth. Overproduction of FprA in *E. coli* resulted in an accumulation of a yellow pigment with the same redox potential and spectral characteristics as flavin (Shimkets, 1990). It seems that FprA is not involved in the flavin biosynthesis pathway. It is more likely that the overproduction of FprA depresses flavin synthesis. The function of the putative gene product in *S. aurantiaca* is not known up to now and remains to be investigated.

Some proteins encoded by the gene of the *csgA* locus might have a toxic effect to *E. coli*. Thus, subcloning of the DNA fragment harbouring the *csgA* locus from the lambda phage DNA into the high copy number vector seemed not to be possible. This would be one explanation for the fact that the fragment harbouring *csgA* could be

subcloned only in a low copy number vector. Or else, *csgA* DNA might bind a factor that is vital for *E. coli* growth and thus inhibits cell growth.

3.2. CsgA protein and similarity with members of the SRD family

The predicted CsgA protein sequence from *S. aurantiaca* contains conserved domains of the short-chain dehydrogenase/reductase (SRD) family. These enzymes share a common function to add or to remove hydrogen from specific substrates using NAD/NADP as a cofactor. Proteins of this family have about 250 amino acids and form usually tetramers. They have a conserved YXXXK motif in the catalytic domain (Enzor and Tai, 1991; Obeid and White, 1992; White et al., 1994). The predicted catalytic triade and its position Ser139, Tyr152 and Lys156 is also more or less conserved upon members of this family (Presson et al., 1991). In the N- terminal part of SRD proteins three glycine residues are highly conserved (Gly14, Gly17, Gly19, the numbering of the *Drosophila* alcohol dehydrogenase) regarding the putative coenzyme binding domain (Persson., et al., 1991).

The predicted CsgA protein in *S. aurantiaca* shows the conserved amino acid sequence in the putative catalytic site (AYAYRMSK) at the carboxyl terminus of the protein. The putative protein contains an AY repeat in this domain as also reported for CsgA of *M. xanthus* (AYAYRMSK) (Lee et al., 1995). The amino acid residues Ser139, Tyr158 and Lys162 of the *S. aurantiaca* CsgA protein seem to form the putative catalytic site. This putative catalytic triad is conserved in the 26 kDa form of CsgA. Also in this case the three glycin residues (Gly7, Gly11, Gly13) would be conserved in the putative coenzyme binding domain of the protein. Thus, the predicted length of 236 amino acids is fits well into the reported average length of about 250 amino acids and position of the conserved domains of this family.

As mentioned above CsgA has a remarkable similarity (about 50%) to many prokaryotic enzymes of this family, including FabG proteins closely related to the SRD family. This family is very large and is involved in a variety of processes like secondary metabolite biosynthesis, transport and catabolism. The FabG protein is highly conserved between bacterial species and is the only known isozyme to catalyze reduction of the □-

keto group. The FabG is a beta-ketoacyl carrier protein reductase involved in fatty acid biosynthesis together with other Fab (fatty acid biosynthesis) enzymes. In *P. aeruginosa* *in vitro* and *in vivo* evidences indicate that the modulation of FabG activity may determine the acyl chain lengths of the homoserine lactones (Hoang et al., 2002). In *P. aeruginosa* two acyl homoserine lactones *N*-(butyryl)-L-homoserine lactone (C₄-HSL) and *N*-[3-oxododecanoyl]-L-HSL (3-oxo-C₁₂-HSL) are required for quorum sensing but bacteria produce also other ALHs with differences in the acyl chain length. Their physiological role is unknown up to know.

3.3. Physiological function of CsgA

In *M. xanthus* CsgA protein is involved in intercellular communication during development. It constitutes intercellular C signalling pathways in which CsgA protein *per se* is the C signal or CsgA protein has enzymatic function to convert some substrate into an actual C signal (Kim and Kaiser, 1990; Kruse et al., 2001; Lee et al., 1994). C signal is necessary for regulation of the cells movement behaviour that results in the formation of rippling waves and aggregation of the cells. Whereas rippling is completely abolished in the *csgA* mutant, aggregation is impaired under certain conditions. Unlike wild type cells, which aggregate into compact translucent mounds after 12 h of starvation on the agar surface, *csgA* mutants aggregate only after 18 h into larger, less compact mounds and ridges (Hagen et al., 1990; Kim and Kaiser, 1990; Shimkets et al., 1983). In a submerged culture the *csgA* mutant cells fail to form any stable multicellular structures (Shimkets et al., 1983). Cells with a mutation in the *csgA* gene are also impaired in sporulation. Moreover, expression of developmental genes that are normally induced after 6 h of starvation is reduced or abolished in *csgA* mutants (Kroos and Kaiser, 1987). Thus, C signalling plays a control role in the regulation of the differential gene expression during *M. xanthus* development.

According to the high homology of about 56 % between *S. aurantiaca* CsgA protein and CsgA of *M. xanthus* and the close phylogenetic relation between these two myxobacteria, it was suggested that CsgA might play a role in the intercellular communication during *S. aurantiaca* development.

The physiological function of CsgA in *S. aurantiaca* was elucidated by inactivation of its encoding gene. The *csgA* gene was disrupted by the insertion of a tetracycline resistance cassette leading to the CsgA knock-out mutant. The mutant exhibits a detectable phenotype during development as a consequence of this inactivation.

Inactivation of *csgA* in *S. aurantiaca* impaired rippling. Mutant cells showed somehow altered migration and aggregation patterns during development, whereas the shape of the fruiting body showed no obvious differences as compared to the wild type. Additionally, mutant cells were differentiated into viable myxospores enclosed in the fruiting bodies. Sporulation seems to be not impaired by the *csgA* inactivation, but the efficiency of sporulation is unknown.

In the wild type *S. aurantiaca* rippling is evident in the early stage of development that precedes aggregation, and it very often accompanies the aggregation stage. Inactivation of the *csgA* gene in *S. aurantiaca* completely abolished rippling of the cells during development. This implies that CsgA protein is required for formation of rippling wave pattern during *S. aurantiaca* development. The role of CsgA protein in the rippling wave pattern formation remains to be investigated. According to the role of the CsgA protein in the regulation of the cells motility behaviour during *M. xanthus* development one may expect more or less similar regulatory pathways in *S. aurantiaca*.

In *M. xanthus* rippling is completely dependent on intercellular C-signalling (Shimkets and Kaiser, 1982). One model proposed that direct end-to-end cell contact initiates C-signalling, which in turn increase the probability that individual cells reverse their direction of gliding maintaining the traveling waves (ripples) (Sager and Kaiser, 1994). Therefore, during rippling cells move about one wavelength and than reverse their gliding direction. The C signal modulates movement pattern of the cells by activation of cytoplasmic Frz proteins by methylation of FrzCD, a methyl-accepting chemotaxis protein (Jelsbak and Sogaard-Andersen 1999; Sogaard-Andersen and Kaiser, 1996; Ward and Zusman, 1997). The Frz proteins regulate the reversal frequency of gliding. Increased methylation of FrzCD is correlated with an increased reversal frequency.

To analyse the effects of the CsgA protein on the motility behaviour of the individual cell during *S. aurantiaca* development it is necessary to perform further experiments. Time laps video microscopy of a single motile wild type and *csgA* mutant cell would reveal effects of CsgA on different motility parameters during development. It would be also necessary to identify the Frz homologous proteins in *S. aurantiaca*. Thus, to investigate the possibility that CsgA protein produces signals that might give an initial input required for activations of Frz proteins whose in turn might regulate reversal gliding frequency of the cells during *S. aurantiaca* development and formation of ripples. To better understand the CsgA dependent modulation of cell behaviour it will be also necessary to analyse the effect of the C-signal in the context of the other cell-cell interactions in *S. aurantiaca*.

Another consequence of the *csgA* inactivation in *S. aurantiaca* was that unlike wild type cells that stay more together in the early stage of development, mutant cells were unable to stay together. Thus, a signal or signals produced by the CsgA protein might contribute in sensing the density of the cells prior to the aggregation stage. Additionally, the *csgA* mutant cells did not respond to stigmolone in a bioassay performed on agar (personal communication with Wulf Plaga). The increase of the aggregation rate was not observed with *csgA* mutant cells in the stigmolone bioassay in contrast to acceleration of the aggregation rate in an assay performed with the wild type cells. As previously mentioned, stigmolone is a novel type of a pheromone substance involved in cell-cell communication in *S. aurantiaca*. It acts in an 1nM range, possibly contributing to cell density sensing (Plaga et al., 1998). Therefore one possibility might be that stigmolone is a substrate for CsgA in *S. aurantiaca*. In that case the reduction of the keto group in stigmolone by CsgA might lead to a real signal molecule, stigmolol, that is exchanged between the cells during development. To investigate this possibility it would be necessary to purify the putative stigmolol from wild type cells. The *csgA* mutant cells would not have stigmolol but the mutant phenotype could be rescued by adding this substance.

However, *S. aurantiaca csgA* mutant cells were able to form fruiting bodies but the localisation of the mature fruiting bodies was altered when compared with the localization of the wild type fruits. Different localisation pattern of the mature mutant

and wild type fruiting bodies was a consequence of an altered cell migration pattern prior to aggregation and formation of the multicellular fruits. The different organisation pattern of the mutant and wild type fruiting bodies was even more serve in the assay performed on filter papers. This might be due to the fact that cells move with different velocities on agar and paper surface because of the different interplays between gliding and adhesion of cells on these two surfaces.

S. aurantiaca forms complex fruiting bodies that consist of a branched stalk bearing several sporangioles, whereas *M. xanthus* forms only mounds filled with spores. Thus, complexity of *S. aurantiaca* fruiting bodies implicates requirements for a more subtle communication network in *S. aurantiaca*. Fruiting body formation process in *S. aurantiaca* probably requires inputs from several independent signalling systems. Therefore, C signalling might not have such an important role in *S. aurantiaca* development as in *M. xanthus*. Moreover, the discovery of SdeK, a histidine kinase, required for expression of some C-signal dependent developmental genes in *M. xanthus* implies that C-signal input is required but it might not be sufficient for *M. xanthus* development (Pollack and Singer, 2001). *M. xanthus sdeK* mutants produce C signal but they are blocked in aggregation and have strong defects in sporulation (Pollack and Singer, 2001).

It is also possible that there is a way to bypass the *csgA* mutational block in *S. aurantiaca* since the pressure to survive under nutrient limitation is high. It might be that mutant cells adopt some suppressor mutations that allow them to bypass the *csgA* block. It is known that overproduction of SocE in *M. xanthus csgA* mutant cells bypasses the *csgA* mutation and cells are able to aggregate and to produce fruiting bodies in the absence of C signalling (Crawford and Shimkets, 2000; Rhie and Shimkets, 1989). However, many compounds may bypass the *csgA* mutational block in *M. xanthus* (Shimkets, et al., 1990). These compounds are normally produced in the *csgA* mutant cells like peptidoglycan (Shimkets and Kaiser, 1982) or glucosamine (Janssen and Dworkin, 1985).

S. aurantiaca mutant cells do not produce CsgA but they are able to respond to it when they are mixed with the wild type cells. Mixed population of cells formed more or

less a wild type localization pattern of mature fruiting bodies on the agar surface. Additionally, when an assay was performed with the mixed population of cells on filter papers some fruiting bodies were still localized in a disperse fashion around the concentrated fruiting bodies in the middle. This organisation pattern can be explained by different ways. It might be that the CsgA protein is anchored in the outer membrane and maybe activated by dimerisation. This can be achieved by direct cell-to-cell contact so that CsgA of one cell interacts with CsgA of another cell and forms an active dimer. Mutant cells do not have CsgA at the cell surface so that no active dimers might be formed by getting in contact to the wild type cells. In this case mutant cells would retain the mutant phenotype when mixed with the wild type cell. Fruiting bodies dispersed around would be build only from mutant cells and fruiting bodies in the middle would be build up from the wild type cells. The rippling waves may be explained as ripples of wild type cells.

Another possibility is that CsgA is an enzyme that produces signal molecules by converting specific substrates into the active signals. Signal molecules induce C signal responses in the recipient cells. In this case mutant cells do not produce but are able to respond to signal molecules. According to this hypothesis fruiting bodies would be build up from wild type and mutant cells together. An indication for this hypothesis was obtained by mixing cells of the *S. aurantiaca* *csgA* mutant with cells of a wild type strain that express the green fluorescence protein. The observed phenotype resulting from this experiment was a wild type fruiting body with an intermediate colour. This suggests that the mutant cells were able to respond to signals produced by wild type cells.

The altered localization of some fruiting bodies of the mixed population is still unclear and requires further investigation. However, it is known that in *M. xanthus* different concentration of CsgA is required for different cellular responses (Kim and Kaiser, 1990; Li et al., 1992). The lowest concentration is rescuing the rippling of the *csgA* mutant cells. Threshold concentrations of CsgA protein in *S. aurantiaca* necessary to initiate different cell responses are not known.

The product of the *csgA* gene is somehow necessary to help the cells to stay tighter together during development and also regulate motility of the cells. It is essential for rippling, a motility behaviour in which cells move in rhythmic oscillations during development. The role of the CsgA protein in these processes is unknown and requires further investigation. According to the remarkable similarity to the members of the SRD family it is possible that CsgA in *S. aurantiaca* has an enzymatic function to produce a signal or signals that act during development.

3.4. Expression of the *csgA* gene in *S. aurantiaca*

The expression of the *csgA* gene in *S. aurantiaca* was determined by qualitative RT-PCR. The *csgA* mRNA was detected in vegetative cells and also during development. Additionally, expression of the *csgA* gene was detected after indol induction in the wild type cells (10, 30, 60 min after adding of the indol).

To estimate the level of *csgA* transcription, a merodiploid mutant containing the *csgA* promoter fused to the *lacZ* reporter gene was constructed. This mutant also contains the wild type *csgA* allele (ATG start of the gene) under the control of about 0,6 kbp of the putative promoter region.

Starvation of the merodiploid cells on water agar resulted in fruiting bodies, which had the same form as the wild type. The mutant was able to ripple and the observed migration and aggregation pattern was the same as in to the wild type. Thus, the 0,6 kbp putative promoter region is sufficient for expression of the *csgA* gene in *S. aurantiaca*. In *M. xanthus* a promoter region of about 0,4 kbp is necessary for optimal *csgA* expression under extreme starvation conditions. A larger upstream region of about 0.9 kbp is necessary for an optimal *csgA* expression in presence of low nutrient levels (Li et al., 1992).

For the determination of *csgA* expression during development β -galactosidase activity was assayed. Further, β -galactosidase activity was determined under stress conditions induced by indol or heat-shock. The quantification of the β -galactosidase

concentration transcribed from the *csgA* promoter was done in an enzymatic assay using a MUG.

No significant β -galactosidase activity was detected in protein extracts (10 μ g) of the vegetative merodiploid mutant. Neither longer starvation, nor indol or heat shock stress induced β -galactosidase activity in the merodiploid strain. A low level of β -galactosidase activity was detected at about 8 h after the beginning of development. Protein extracts from the wild type after the same time course of development were used to estimate the background level in the fluorometric assay.

In the fluorometric assay with five times more protein low levels of the β -galactosidase activity was detected in the vegetative cells and at the different stages of development in merodiploid mutant. The level of the β -galactosidase activity slightly increased at the beginning of development, and reached its maximum at about 8 h after the beginning of starvation. A low level of the β -galactosidase activity was detected 30 min after indol and heat-shock treatment. As control of the background signal the protein extract (50 μ g) from wild type cells after the same time course of development was used.

Since no significant β -galactosidase activity was detectable in developing cells when 10 μ g of protein extract was used and low level of the β -galactosidase activity was observed when five times more total protein was used it is suggested that *csgA* is expressed at very a low level during development. The, low level of β -galactosidase activity might be due to a low strength of the *csgA* promoter.

Additionally, β -galactosidase is a cytoplasmatic protein and CsgA of *S. aurantiaca* is supposed to be an extracellular protein. The degree to which the reporter protein concentration mimics the concentration of CsgA in *S. aurantiaca* is unknown.

β -galactosidase was detected in an *in situ* assay. The stems of the fruiting bodies of the merodiploid mutant were stained blue when X-gal was added to the starvation agar.

Under these conditions wild type fruiting bodies were orange. Since this assay is not quantitative, the level of β -galactosidase expression cannot be elucidated.

Determination of the *in situ* beta galactosidase activity indicates that the *csgA* gene is expressed during development in *S. aurantiaca*. This result is consistent with the results of the RT-PCR. Since the level of the *csgA* expression appears to be low it would be necessary to use some more sensitive techniques like real-time PCR or a chemiluminescence assay that has a much higher sensitivity than the conventional fluorometric assay.

3.5. Immunological identification of CsgA in *S. aurantiaca*

To study the production of CsgA protein in the course of *S. aurantiaca* development, antisera against two peptides derived from CsgA and against a recombinant protein containing a part of CsgA were raised. Both peptide sequences are enclosed in the sequence of the recombinant protein (6xHis-CsgA-DHFR). Purified recombinant protein was used as a positive control in Western blot analysis using the anti-peptide-antisera or anti-6xHis-CsgA-DHFR antibodies. Both antisera recognized the purified recombinant protein, but CsgA protein was not detected in crude protein extracts isolated from *S. aurantiaca* cells during vegetative growth or development. Total protein was extracted from the cells by boiling in SDS, or by lysis of the cells with urea and CHAPS for solubilisation.

The sensitivity of both sera was tested by titration of various concentrations of the recombinant protein. The sensitivity was in the nanogram range. The anti-peptide-antibodies had higher sensitivity (3 ng) than anti-6xHis-CsgA-DHFR-antibodies.

According to these results the amount of CsgA protein in total protein extracts isolated from 1×10^8 or 1×10^9 cells is expected to be less than 1 ng if the molecular mass is assumed to be 26 kDa. Thus, expected number of CsgA protein molecules per cell is less than 100. This indicates that *csgA* transcription is low. This is consistent with the

results of quantification of the *csgA* transcription. A further possibility could be that *S. aurantiaca* CsgA may be very unstable protein.

In *M. luteus* a full-length CsgA form of about 25 kDa was detected in vegetative cells and during development. The smaller form of about 17 kDa was detected during development starting 3 h after the beginning of starvation (Kruse et al., 2001). In *M. luteus* the zwitterionic detergent CHAPS was found to solubilize CsgA (17 kDa) (Kim and Kaiser, 1990). CsgA (17 kDa) appears to be bound to the cell envelope because it sediments with the membrane fraction of wild type cells and must be extracted from the membrane fraction with a detergent (CHAPS) (Kim and Kaiser, 1990). *M. luteus* CsgA is found near the cell surface in the extracellular matrix as detected by localization of the immuno-gold labelled antibody against CsgA (Rafiee and Shimkets, 1990). Additionally, in *M. luteus* was estimated that the number of CsgA molecules per developing cell is between 1,100 to 2,200 (Shimkets and Rafiee, 1990).

3.6. Perspectives

The understanding of intercellular communication in *S. aurantiaca* is just at the beginning. To better understand the physiological role of CsgA protein in intercellular signalling the following investigation should be carried out.

Identification of the real translational start of the gene by insertional mutagenesis upstream of the putative start codons or site-direct mutagenesis of all three putative start codons. Examination of the mutant phenotype during development would reveal the real start of the gene.

Identification of the transcriptional start of the *csgA* gene by primer extension that should be carried out with the total RNA isolated from cells after 8 h of development.

Quantification of the *csgA* transcription during development with a highly sensitive method such as real-time PCR.

To investigate cellular localization of the native CsgA protein it would be necessary to perform immuno-gold labeling with anti-CsgA-antibodies followed by transmission electron microscopy.

To investigate the possibility that CsgA has an enzymatic function to convert some substrate into actual signal molecule or molecules it would be necessary to identify the putative substrate. Additionally, importance of the proposed catalytic triad for CsgA function should be analysed by performing single amino acids substitution.

In order to understand transmission of CsgA generated signals isolation and characterization of the putative receptor protein should be performed.

Time laps video microscopy of a single motile wild type and *csgA* mutant cells to reveal effects of CsgA protein on different motility parameters during development.

To fully understand the CsgA dependent modulation of cell behaviour it will be also necessary to analyse the effect of the C-signal in the context of other cell-cell interactions in *S. aurantiaca* during the complex developmental process.

IV. Materials and Methods

4.1. Materials

4.1.1. Specified chemicals, consumables and equipments

Bulk chemicals and laboratory articles were obtained from the following companies:

Becton, Dickinson & Co., Sparks, USA
 Carl Roth GmbH & Co., Darmstadt
 Mallinckrodt Baker B. V., Deventer, Holland
 Merck, Darmstadt
 NeoLab, Heidelberg
 Roche Diagnostics GmbH, Mannheim
 Serva Feinbiochemika GmbH, Heidelberg

4.1.1.1. Chemicals

Agarose	Sigma
Bacto Agar	Difco
Bacto Trypton	Difco
Bacto Agar	Becton
Bacto Trypton	Becton
Bovine Serum Albumin (BSA)	Serva
5-Brom-4-Chlor-3-Indolyl- β -D-Galaktopyranosid (X-Gal)	Biomol
Bromphenol-blue	Chroma
Casiton	Difco
Complete TM	Roche
Diagnostics	
Diethylpyrocarbonat (DEPC)	Sigma
Dinatrium 3-(4-methoxyspiro {1,2-dioxetan-3,2'-(5'-chloro)	
Tricyclo [3.3.1.1 ^{3,7}]decan} -4-yl) phenylphosphat (CSPD)	Roche
Diagnostics	
Dithiothreitol (DTT)	Serva
Etylenedinitrilo tetraacetic acid (EDTA)	Serva
Guanidine hydrochloride (GuHCl)	Gerbu
Indole	Sigma
Isopropanol	Merck
Isopropyl thiogalactoside (IPTG)	Sigma
Low melting point agarose	Sigma
4-Methylumbelliferyl β -D-galactoside (4-MUG)	Sigma
Nitro blue tetrazolium (NBT)	Sigma
N-2-Hydroxyethylpiperazin-N'-2 ethanesufonic acid (HEPES)	Roth
N, N, N', N'-Tetramethyl ethylerendiamine (TEMED)	Serva
Phenylmethlsulfonyl fluoride (PMSF)	Sigma
Xylene cyanol FF	Serva

4.1.1.2. Consumables

Biodyne A, B blotting membranes	Pall
Glass beads (0,1 mm)	Serva
Immobilon- P (PVDF membrane)	Millipore
Nitrocellulose membrane	Appligene
Hyperfilm-ECL (RPN 2103)	
X-ray film	Kodak/Fuji

4.1.1.3. Equipment

ÄKTA™	Pharmacia Biotech
Digitale Kamera Olympus DP10	Olympus
GenePulser (Pulse Controller)	Bio-Rad
Micromicroscope M 420	Leica
Semi-dry protein blotter apparatus	Pegasus
Sonifier B15	Branson
Thermocycler T3-thermoblock	Biometra
Ultracentrifuge L8-70M	Beckman
UV-crosslinker	Stratagene
Vac-Man® laboratory vacuum manifold	Promega
Vacuum blotter	Appligen
Speed-vac-concentrator Savant	Bachofen
Spectrofluophotometer RF 500	Shimadza
Spectrophotometer	Kontron

4.1.2. Protein

4.1.2.1. Antibodies

Anti Dehydrofolate reductase ZMBH	Prof.Dr. Herrmann,
Anti rabbit IgG, alkaline phosphate-conjugated	Dianova
Anti rabbit IgG, peroxidase-conjugated	Dianova

4.1.2.2. Protein weight standards

SDS-PAGE Molecular Weight Standards, Broad Range	Bio-Rad
Precision plus protein™ standards, all blue	Bio-Rad

4.1.3. Reagent kits for methods in molecular biology and enzymes

4.1.3.1. Reagent kits for methods in molecular biology

Access RT-PCR system	Promega
Bio Rad Protein Assay	Bio Rad
Biotin Luminescent Detection Kit	Roche Diagnostics
DNA-free™ Kit	Ambion
Gigapack® III gold packaging extract	Stratagene
Lamda DASH® II/BamHI Vector kit	Stratagene
Minelute PCR purification kit	Qiagen
Nucleospin plasmid purification kit	Macherey & Nagel
Nucleobond plasmid purification kit	Macherey & Nagel
QIAquick PCR Purification kit	Qiagen
QIAquick Gel Extractions kit	Qiagen
Wizard lambda preps DNA purification system	Promega

4.1.3.2. Enzymes

AMV Reverse Transcriptase	Promega
Alcaline phosphatase, calf intestinal	New England Biolabs
Ampli Taq DNA polymerase	Perkin Elmer
Biotin High Prime	Roche Diagnostics
Deep Vent DNA polymerase	New England Biolabs
Proteinase K	Roche Diagnostics
Restriction endonucleases	New England Biolabs
Shrimp alkaline phosphatase	Roche Diagnostic
Taq DNA polymerase	Roche Diagnostic
T4 DNA ligase	Promega
	Roche Diagnostics

4.1.4. Nucleic acids

4.1.4.1. Plasmid

litmus 28	Biolabs
mini Tn5 lacZ1	(de Lorenzo et al., 1990)
pACYC177	Biolabs
pBC SK+/-	Stratagene
pBS SK+/-	Stratagene
pBS II SK+/-	Stratagene
pBR 322	Biolabs
pQE42	Qiagen
pUC4 KIXX	(Barany, 1985)
pUC18	(Yanish-Perron et al., 1985)

4.1.4.2. Primers

All the primers were synthesized by MWG-Botech AG.

pACYC177s	CCATATTCAACGGGAAAC
pACYC177as	CCATATAAATCAGCATCC
<i>csgA7</i>	CTGGATGTGCTCATCAAC
<i>csgA7/Bgl II</i>	GAAGATCTTCCTGGATGTGCTCATCAAC
<i>csgA8</i>	CTGGAAGTCGAGGAACATG
<i>csgA12</i>	CTGGTTGGTGTGTCGAGC
<i>csgA13</i>	CAGCTTCCTTGACAGAGAAC
<i>csgA14</i>	CTTTCTTCACAACCTCAC
<i>csgA15</i>	CATCCGTTCTGGCTTCGC
<i>csgA16</i>	CTGCTCCTTGGTGTGTC
<i>csgA17</i>	CTGGTTGGTGTGTCGAGCC
<i>csgA20</i>	CATGAAGGAGCACCCTTGC
<i>csgA20XbaI</i>	GCTCTAGACATGAAGGAGCACCCTTGC
<i>NotI csgA21</i>	ATAAGAATGCGGCCGCCAGCTGCTTCAGCGTGGG
pQE42 forward	CAGGAGGAAAAAGGCATC
pQE42 reverse	CTAGCTTGGATTCTCACC
TcfwXba	GCTCTAGACTCATGTTTGACAGCTTAT
TcrvXba	GCTCTAGAAGTTCTCCGCAAGAATTGATTGGC

4.1.4.3. Oligonucleotides

Set of dATP, dCTP, dGTP, dTTP Roche Diagnostic

4.1.4.4. DNA and RNA molecular weight markers

100 pb DNA ladder New England Biolabs
DNA molecular weight marker IV Roche Diagnostic
RNA marker I Roche Diagnostic

4.1.5. Bacterial strains

4.1.5.1. *Escherichia coli*

ABLE C	<i>lac(LacZw)</i> [Kan ^r Mcr ⁻ McrCB- McrF- Mrr- HsdR(<i>rk-mk-</i>)] [F' <i>proAB lacIqZ</i> M15 Tn10(Tet ^R)]
ABLE K	<i>lac(LacZw)</i> [Kan ^r Mcr ⁻ McrCB- McrF- Mrr- HsdR(<i>rk-mk-</i>)] [F' <i>proAB lacIqZ</i> M15 Tn10(Tet ^R)]
DH5 \square	F' <i>endA1 hsdR17(r_{k-} m_{k+})glnV44thi-1recA1gyrA(Nal^r)</i> <i>relA1</i> \square (<i>lacIZYAargF</i>)U169 <i>deoR</i> (\square 80 <i>dlac</i> \square (<i>lacZ</i>)M15)
XL1-Blue MRA (P2)	D(<i>mcrA</i>)183 D(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1</i> <i>gyrA96 relA1 lac</i> (P2 lysogen)
XL1-Blue MRF'	D(<i>mcrA</i>)183 D(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1</i>

recA1 gyrA96 relA1 lac[F' proAB lacIqZDM15 Tn10

(Tet^R)]

M15 (pREP4) Nals, Strs, Rifs, *lac-*, *ara-*, *gal-*, *mtl-*, *F-*, *recA+*, *uvr+*.
(Qiagen)

4.1.5.2. *S. aurantiaca* strain

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

4.1.6. Media and stocks solutions

4.1.6.1. Media

LB medium (pH 7.2)	1% Bacto tryptone; 0,5% Bacto yeast extract, 1% NaCl
SOC (pH 7.0)	2% Bacto tryptone; 0,36% Glucose; 0,5% Bacto yeast extract 0,019% KCl; 0,2% MgCl ₂ ; 0,25% MgSO ₄ ; 0,059% NaCl
CY (pH 7.2)	0,3% Casitone; 0,1% Bacto yeast extract; 0,1% CaCl ₂ x 2 H ₂ O
Tryptone medium (pH 7.2)	1% Bacto tryptone; 0,2% MgSO ₄ x 7H ₂ O
Water agar medium	0.1% CaCl ₂ ; 1,5% Bacto agar

4.1.6.2. Buffers and stock solutions

Antibiotics stock solutions

Ampicillin sodium salt 100 mg/ml in H₂O
Kanamycin sulphate 50 mg/ml in H₂O
Oxi- tetracylin 7,5 mg/ml in DMSO
Tetracycline hydrochloride 10 mg/ml in 100% Ethanol
Streptomycin sulphate 125 mg/ml in H₂O

Ethidium bromide solution (20,000 x) 10 mg/ml in H₂O
IPTG stock solution 100 mM in H₂O
X-gal stock solution 20 mg/ml in dimethylformamid

Enzyme buffer:

- 10xA, B, H, L, M buffer Roche Diagnostics
- 10xNEB 1, 2, 3, 4 buffer New England Biolabs
- 5xAMV buffer Promega
- 10 x Ligase buffer Roche Diagnostics
- 10xRNase buffer Roche Diagnostics

Hepes buffer (pH 7.2) 100 mM Hepes, 10 mM CaCl₂

SM (phage suspending buffer) 0,58 (w/v) NaC; 0,2 (w/v) MgSO₄x7H₂O
50 mM Tris-HCl (pH 7,5); 2 % gelatin solution

20xSSC (pH 7.0)	3 M NaCl; 0.3M Sodium citrate
TE buffer	10 mM Tris-HCl, pH 8.0; 1 mM EDTA
50 xTAE buffer (pH 8)	2 M Tris-acetate; 0.05 M EDTA

4.2. Methods

4.2.1. Microbiologic techniques

4.2.1.1. Growth of *E. coli*

E. coli strains were grown in liquid LB or SOC medium at 37°C with vigorous shaking at 170 rpm. Growth on solid LB medium (1,5 % agar) was performed over night at 37°C. Appropriate antibiotics were added using the concentrations described above. Storage of the LB plates was possible at 4°C for about 14 days.

4.2.1.2. Growth of *S. aurantiaca*

S. aurantiaca strains were grown in liquid Tryptone medium (with appropriate antibiotics) at 32°C with vigorous shaking (130 rpm). For inoculation of new cultures a minimum of 10⁶ cells/ml is necessary otherwise the culture does not start to grow. After transformation the selected clones were inoculated first in a small volume (about 3 ml) to make them grow and latter transferred into larger cultures. The doubling time in the logarithmic phase is about 7 hours. After the cells reach the stationary phase they die quickly.

It is not possible to store *S. aurantiaca* cultures at 4°C.

4.2.1.3. Indol induced sporulation of *S. aurantiaca* (Gerth and Reichenbach, 1994)

S. aurantiaca spore formation can be separately induced from the fruiting body formation by addition of 0,5 mM indol in the logarithmic cultures (2x10⁸ cells/ml). Incubation followed at 32°C with vigorous shaking (130 rpm).

4.2.1.4. Heat shock of *S. aurantiaca*

S. aurantiaca strains were grown in liquid Tryptone medium at 28°C until logarithmic phase was reached (2x10⁸ cells/ml). Then the culture was shifted to 37°C and aliquots were taken after different times for further analysis.

4.2.1.5. *S. aurantiaca* fruiting body formation assay

To initiate fruiting body formation *S. aurantiaca* strains were grown in liquid Tryptone medium at 32°C until logarithmic phase was reached (2×10^8 cells/ml). Cells were sedimented by centrifugation at 5000 rpm for 15 min at 4°C. The cell pellet was washed twice in 100 mM HEPES buffer. The cells were resuspended in washing buffer to a concentration of 4×10^8 cells/ml. Portions of 5 μ l (2×10^8 cells/ml) were spotted on the surface of starvation agar plates that were dried before. Plates were dried about 5 min and incubated at 32°C. After different times the fruiting body formation was controlled under the microscope. The development of the wild type takes about 24 hours.

Additionally the described fruiting body formation can be performed on filter papers that were placed on the surface of the starvation agar. These filter papers can be dried after an inoculation of about 10 days in a desiccator (duration about one week). Long-term storage (years) of the filter papers is possible in sterile glass tubes.

4.2.1.6. Germination of *S. aurantiaca* spores

To test the germination ability of spores from various *S. aurantiaca* strains, prepared filter papers (4.2.1.5.) were placed upside down onto a CY agar plate and incubated two days at 32°C. Then a two-day incubation of the turned filter paper followed. The ability to germinate can be observed under the microscope as swarming vegetative cells become visible.

4.2.1.7. Preservation of *E. coli* cultures

E. coli strains were grown in liquid LB medium until logarithmic phase was reached. Sterile glycerol was added to a final concentration of 20 %. Storage is possible at -80°C.

4.2.1.8. Preservation of *S. aurantiaca* cultures

S. aurantiaca was grown in liquid Tryptone medium at 32°C until logarithmic phase was reached. About 1,5 ml of culture was sedimented by centrifugation at 5000 rpm for 15 min at 4°C. The pellet was resuspended in 0,5 ml Tryptone medium and sterile glycerol was added to a final concentration of 20%. The storage of *Stigmatella* is only possible at -80°C or in liquid nitrogen. Long term storage on filter papers is possible for years (4.2.1.5.).

4.2.1.9. Electroporation

This transformation technique is a highly efficient (about 10^{10} recombinant clones/ μ g plasmid DNA) and fast method to transform prokaryotic cells as first described by Shigekawa and Dower, 1988. Short induced high voltage causes the reversible formation of pores in the cell membrane where the DNA can pass through.

4.2.1.9.1. Electroporation of *E. coli*

The *E. coli* strain for transformation was grown in 250 ml SOC medium at 37°C with vigorous shaking until the culture reached the OD of about 0.6 at 600 nm. After chilling down on ice for 30 min, cells were sedimented by centrifugation at 5000 rpm for 15 min at 4°C. The cell pellet was washed twice with 125 ml cold sterile water followed by centrifugation at 5000 rpm for 15 min at 4°C. The cell pellet was resuspended in 10% ice-cold sterile glycerol to a final volume of 2-3 ml. Aliquots of 50 μ l were frozen in liquid nitrogen and stored at -80°C.

For electroporation about 50 ng of plasmid DNA was added to 50 μ l of the electroporation prepared *E. coli* cells and placed on ice. The mixture was transferred into a cold electroporation cuvette (Gap distance 1 mm). The cuvette was placed in the BioRad Gene Pulser with following conditions: field strength 12,5 kV/cm, electric capacity 25 μ F, resistance 200 Ω . Immediately after electroporation 1 ml of SOC medium was added in the cuvette to resuspend the cells. The cell suspension was transferred into an Eppendorf tube and incubated at 37°C for one or two hours depending on the antibiotic resistance of the transformed plasmid. After incubation various aliquots of cells were plated onto LB agar plates with appropriate antibiotics.

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

S. aurantiaca cells were grown in liquid Tryptone medium at 32°C until logarithmic phase was reached (2×10^8 cells/ml). The culture was sedimented by centrifugation at 5000 rpm for 15 min at 20°C. The cell pellet was washed twice in 5 mM HEPES and centrifuged at 5000 rpm for 15 min at 20°C. Finally the cell pellet was resuspended in the same buffer to a final concentration of 4×10^{10} cells/ml. The *S. aurantiaca* cells were placed on ice. A portion of 40 μ l of cells was mixed with about 0,5 μ g of plasmid DNA. Electroporation was performed in electroporation cuvette (Gap distance 1mm) in the BioRad Gene Pulser with following conditions: field strength 0,85 kV/cm, electric capacity 25 μ F, resistance 200 Ω . Immediately after electroporation 1 ml of Tryptone medium was added in the cuvette to resuspend the cells. The cells were transferred into 50 ml Tryptone medium supplement with streptomycin sulphate and incubated overnight at 32°C. The cells were sedimented by centrifugation at 5000 rpm for 15 min at 4°C and resuspended in 2 ml 100 mM HEPES buffer. Different portions of cells were mixed with 3 ml (42°C) soft agar (Tryptone medium with 0.75% agar) and plated onto Tryptone agar plates. Appropriate antibiotics were added to the soft agar and to the agar plates. The incubation was performed at 32°C and the time of incubation strongly depends on the antibiotic resistance.

4.2.1.10. Blue white colony screening selection of *E. coli*

Many of the plasmid vectors contain an *E. coli* DNA fragment with the regulatory sequence and the coding part of the *lacZ* gene. This DNA fragment encodes the amino terminal part of the enzyme β -galactosidase. This type of the plasmid is propagated in host cells that express the carboxy terminal part of β -galactosidase. The active enzyme hydrolyses X-gal producing an insoluble blue colour.

The polylinker fragment of these kinds of plasmids is inserted in-frame to the beginning of the coding region of the *lacZ* gene. Insertion of foreign DNA into the polylinker inactivates the gene and abolishes β -galactosidase activity. Bacteria carrying recombinant plasmid DNA form white colonies in presence of X-gal.

To identify *E. coli* clones with the recombinant plasmid DNA specific selection plates were used. Selection plates were prepared by spreading the chromogenic substrate X-gal (2%) mixed with IPTG (20%) on the surface of the LB agar. After transformation, several aliquots were put on the surface of these plates and the plates were incubated at 37°C over night. To develop the blue colour incubation at 4°C for several hours follows.

4.2.1.11. Preparation of bacteriophage host *E. coli* cells

As a host strain for lambda phage infection XL1 Blue MRA (P2) strain was used. XL1 Blue MRA (P2) is a P2 lysogen of XL1 Blue MRA strain, that allows only grow of recombinant phages. In recombinant phages *red* and *gam* genes are replaced with the insert DNA and therefore they can grow in the (P2) lysogenic strain. LB medium supplemented with 10 mM MgSO₄ and 0,2% maltose was inoculated with 1/100 volume of a fresh over night culture of *E. coli* strain XL1 Blue MRA (P2) and incubated at 37°C with vigorous shaking until the culture reached the OD 1,0 at 600 nm. The cells were sedimented by centrifugation at 5000 rpm, for 10 min at 4°C. The pellet was resuspended in sterile 10 mM MgSO₄ to a final OD of 0.5 at 600 nm. Prepared host cells were used immediately for infection with the lambda phage.

4.2.1.12. Infection of *E. coli* with the bacteriophages

A portion of 600 μ l of the *E. coli* cells (preparation 4.2.1.11.) was mixed with the different dilutions (1:10; 1:100; 1:1000) of lambda phages in the SM buffer. The mixture was incubated at 37°C for 15 min to allow phages to attach to the host cells. After incubation, infected host cells were mixed with 7 ml LB top agar (0,7% agar, 48°C) and plated on the LB agar plates (1.5% agar, 150 mm diameter). The plates were incubated at 37°C for about 9h until the phage plaques reached a size of about 1 mm.

4.2.1.13. Tittering of the phage library

To estimate the number of phages in the constructed phage library a tittering reaction was performed. Host cells were prepared as described (4.2.1.11.) and portions of 200 μ l of cells were infected with the different dilutions of phages in SM buffer (1:10; 1:100; 1:1000). The mixtures were incubated at 37°C for 15 min. About 3 ml pre-warmed LB top agar (48°C) was added in the mixture and plated immediately on LB agar plates (7,5 mm diameter). After incubation at 37°C for about 9 h the plaques were counted and the phage titter - plaque forming units per millilitre (pfu/ml) was determined.

4.2.1.14. Amplification of the *S. aurantiaca* genomic phage library

S. aurantiaca genomic library was amplified in order to make a stable high titer stock for long time storage at -80°C . *E. coli* XL1-Blue MRA (P2) host cells were prepared as described (4.2.1.11.). A portion of 600 μl of cells was mixed with aliquots of the SM buffer containing about 5×10^4 pfu/ml. The mixture was incubated at 37°C , for 15 min. After incubation the infected host cells were mixed with 7 ml (48°C) LB top agar and plated on the LB agar plates (150 mm diameter). The plates were incubated at 37°C for about 9h. The plates were covered with 8 ml SM buffer and shake gently over night at 4°C . SM buffer with bacteriophages were transferred into a Falcon tube. The bacteriophage suspension was mixed with chloroform to a final volume of 5% (v/v) and incubated at room temperature for 15 min. After a centrifugation at 5000 rpm for 10 min the supernatant was transferred into a new Falcon tube and mixed with chloroform to a final concentration of 0,3% (v/v). Storage at 4°C is possible for about one month. For long time storage DMSO (7% v/v) was added to the amplified library and aliquots were stored at -80°C .

4.2.1.15. Plaque lifts

Fresh host cells were prepared (4.2.1.11.), infected with phages and plated on large LB plates (150 mm diameter) to obtain 50 000 pfu/plate. After incubation at 37°C , about 9 h, plates were pre-cooled at 4°C for several hours. A nylon membrane (Byodine A 1,2 mm) was put on the surface of the LB agar plates for about two min. The orientation of the nylon membrane should be fixed. Membranes were placed on the surface of filter paper that is saturated with a denaturation solution (1,5 M NaCl, 0,5 M NaOH). After two min the membranes were neutralized for 5 min (on filter paper saturated with 1,5 M NaCl, 0,5 M Tris- HCl pH 8), washed (filter paper with 0,2 M Tris-HCl pH 7,5) and transferred to 2xSSC. After drying of the membranes on Whatman paper the DNA was crosslinked 2 times in a UV crosslinker.

4.2.1.16. Purification of bacteriophage clones

After the screening procedure several rounds of purification of positive recombinant phage was performed. Positive phage clones were picked from the plate transferred in 1ml SM buffer and incubate at room temperature for 1h. The phage titer was estimated and several rounds of host cells infection, plating and hybridisations were performed until each plaque on the plate correspond to the dark spot on X-ray film. Purified phages were dissolved in SM buffer with 7% DMSO and stored at -80°C for long time.

4.2.2.3. Isolation of lambda DNA

The Wizard Lambda Preps DNA Purification System (Promega) was used for purification of lambda DNA from plate culture lysates. Bacteriophages are propagated in bacteria grown on soft agarose instead of soft agar to avoid polyanionic contaminants that can inhibit subsequent enzymatic reactions. The isolation procedure was accomplished following the protocol supplied by manufacturers. The lambda DNA was eluted from the purification resin with the TE buffer and stored at -20°C.

4.2.2.4. Phenol/chloroform extraction of DNA (Sambrook et al., 1989)

A standard procedure was used to remove proteins from DNA solutions. An equal volume of TE saturated phenol (pH 7) was added to the DNA solution. After mixing of the solution and centrifugation at 13 000 rpm for 15 min at room temperature, the upper aqueous phase containing the DNA was carefully transferred into a new tube. Extraction was repeated once with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) and once with an equal volume of chloroform/isoamylalcohol (24:1). DNA was precipitated with isopropanol or ethanol, dried and dissolved in TE buffer.

4.2.2.5. Alcohol precipitation of DNA

DNA was precipitated from solutions using various combinations of salt and alcohol. This is the most common method used to concentrate DNA or to precipitate DNA from the aqueous phase after phenol/chloroform extraction. A 0,1 volume of 3M sodium acetate (pH 5,2) or 0,1 volume of 8 M lithium chloride was added to the DNA sample followed by the addition of 2,5 volumes of 98% ethanol (at -80°C, 1-2 h) or equal volume of isopropanol (at room temperature, for 30 min). DNA forms complexes with the salt that reduces the solubility in alcohol. Further, DNA was washed with 0,7 volume 70% ethanol, dried and dissolved in TE buffer.

4.2.2.6. Quantitation of DNA

To estimate the concentration and purity of DNA samples a spectrophotometric method was used. Concentration of DNA was calculated by the OD value at 260 nm:

DNA concentration [g/ml] = absorbance at 260 nm x dilution factor x extinction coefficient (extinction coefficient of DNA is 50)

Information about purity of the sample is estimated by calculating a so-called OD₂₆₀:OD₂₈₀ ratio. A pure sample of DNA has a OD₂₆₀: OD₂₈₀ ratio of about 1,8.

4.2.2.7. DNA restriction

DNA restrictions were performed according to standard protocols (Sambrook, *et al.*, 1989). The restriction conditions were chosen depending on the used enzyme as recommended by the manufacturer. To inactivate the enzyme reaction several methods were used: heat inactivation, phenol extraction or adding of EDTA (20 mM final concentration).

4.2.2.8. Partial digestion of DNA

To obtain partial cleavage of genomic DNA, a set of serial dilutions of enzyme were used as set up in the following way. 70 μ l of a DNA solution (70 μ g) in 1x appropriate enzyme buffer was divided so that each Eppendorff tube contained 10 μ l of the DNA solution except the first one that contained 20 μ l. The appropriate restriction enzyme was added in first tube followed by careful mixing. Then portions of 10 μ l were transferred from one tube to the next. Last tube was used as a negative control so diluted enzyme was not added in it. All tubes were incubated for a defined time at the appropriate temperature. The enzymatic reactions were stopped by heating and checked by agarose electrophoresis.

4.2.2.9. DNA ligation

Ligation of double-stranded DNA was performed using T₄ DNA ligase. This enzyme catalyses the formation of phosphodiester bonds between 3' hydroxyl and 5' phosphate ends in double stranded DNA using ATP. 1 U of T₄ DNA ligase was used to ligate insert and vector DNA (molar ratio 3:1) in a 20 μ l reaction containing 1x ligation buffer at 15°C over night. T₄ DNA ligase was inactivated by incubation at 65°C for 10 min or by addition of 2 μ l 0,5 M EDTA (pH 8). After inactivation 28 μ l sterile water was added and 5 μ l of the ligation reaction was used for electroporation of *E. coli* strains.

4.2.2.10. Construction of the *S. aurantiaca* phage library

The ligation reaction was performed using about 0,4 μ g of the chromosomal DNA of *S. aurantiaca* (9-23 kbp) and 1 μ g of the Lambda DASH II (predigested with *Bam*HI) vector arms. The reaction mix was supplemented with 10 mM rATP (pH 7,5), 2U of T₄ DNA ligase (Stratagene) and incubated overnight at 4 °C. Portions of 2 μ l or 3 μ l of the ligation reaction were packed into bacteriophage particles *Bam*HI/Gigapack III Gold (Stratagene). The titer of both libraries was estimated as described in the method part and appeared to be about 1×10^5 pfu/ml before and about 1×10^8 pfu/ml after the amplification of the phage libraries.

4.2.2.11. Filling of 5' overhanging ends of DNA

For many cloning experiments, it is necessary to convert 5' overhanging ends generated by restriction with some enzymes into blunt ends. For this purpose Klenow enzyme was used. Klenow enzyme is the large fragment of DNA polymerase I with a 5'-3' polymerase activity but lacks the 5'-3' exonuclease activity. The enzyme catalyses the addition of mononucleotides from desoxynucleoside-5'-triphosphates to the 3-hydroxyl terminus of the template DNA.

A 10 μ l reaction contained 0,1 mM of each of the dNTPs, 0,1-4 μ g DNA and 1 U of the Klenow enzyme per μ g DNA. Incubation was performed at 37°C for 30 min. The enzyme was inactivated by heating the reaction mix up to 75°C, for 10 min or by adding 1 μ l of 0,5 M EDTA. DNA was extracted by the phenol method or purified on column.

4.2.2.12. Removing of 3' overhanging ends of DNA

For many cloning experiments, it is necessary to remove 3' overhanging ends of DNA fragments. Removing of 3' extensions is carried out using the 3'-5' exonuclease activity of the T₄ DNA polymerase. In the presence of high concentrations of all four dNTPs the enzyme removes 3' extensions and generates blunt ends.

In a 25 μ l reaction 1x buffer A (Roche Diagnostics) supplement with 0,05 mM of each of the dNTPs, 2,5 μ g BSA, 2 μ g DNA and 10 U of T₄ DNA polymerase were mixed. Reaction was carried out at 11°C for 15 min. T₄ DNA polymerase was inactivated by heating at 75°C for 10 min or by adding 1 μ l of 0,5 M EDTA. DNA was extracted by phenol or purified on column.

4.2.2.13. Dephosphorylation of DNA fragments

In order to prevent self-ligation of vector DNA during cloning reactions it is necessary to remove the phosphate residue from the 5' end of the DNA using alkaline phosphatase. Phosphorylated insert DNA can be ligated to the vector DNA more efficiently. In a 10 μ l reaction 1 x dephosphorylation buffer, 100 ng of DNA and 1 U of shrimp alkaline phosphatase were added. Incubated followed at 37°C for 60 min in the case of blunt end DNA fragments or at 37°C for 10 min in case of sticky end DNA fragments. In order to inactivate the enzyme activity the reaction mix was heated up to 65°C for 15 min.

4.2.2.14. Amplification of DNA fragments *via* PCR (polymerase chain reaction)

The polymerase chain reaction (PCR) is a method for rapid amplification of DNA molecules *in vitro* with a thermostable DNA polymerase. As enzymes for amplification Taq DNA polymerase was used that has no proofreading or Deep Vent Polymerase that has proofreading function. The primers (20-30 bp) were constructed in a way so that they form no internal loop structures. If the primers were used for amplification of fragments for directional cloning then specific restriction site were added at the 5' end of the primers.

In a 100 μ l PCR reaction 1x polymerase buffer, 2,5 mM of each dNTP, 500 ng of chromosomal DNA or 100 ng plasmid DNA, 50 pmol of each primer and 1-2 U DNA polymerase were mixed. In general 25-30 PCR cycles were performed:

Initiation denaturation was performed at 95 °C for 10 min.

Following denaturations steps at 95°C for 1 min.

Annealing was performed for 1 min at temperatures between 50 - 68 °C according previously calculated value for each primer pair, used for PCR.

Extension step was carried out at 72 °C for 1 min and 30 seconds. Amplified DNA was purified on PCR purification column. As controls PCR reactions without template DNA or without primers were performed in parallel.

4.2.2.15. Purification of PCR products

In order to purified DNA fragments amplified by PCR the QIAquick PCR Purification Kit (Qiagen) was used. DNA fragments ranging from 100 bp to 10 kbp were purified

from primers, nucleotides, polymerases and salt on a special silica gel membrane. The protocol recommended by the manufacturer was used for purification.

4.2.2.16. DNA sequencing

Sequencing of the DNA was performed with ABI Prism™ 377 Sequence System (Perkin-Elmer Corporation) in the sequence facilities of the ZMBH. For sequencing the standard method described by Sanger (Sanger *et al.*, 1977) was used.

4.2.3. Electrophoresis of DNA

4.2.3.1. Agarose gel electrophoresis of DNA

The electrophoresis was accomplished by horizontal gel system. Between 0,8-1,5 % agarose in 1 x TAE buffer was used as gel matrix depending of the fragment sizes that were separated. The DNA sample was mixed with 1/5 volume of 5xGLB buffer and loaded on the gel. Gel electrophoreses was performed at 1-5 V/cm in 1x TAE buffer. After electrophoresis the gel was stained with 0,5 µg/ml ethidium bromide for 5 min and the DNA bands were visualized on a transilluminator with UV (254 nm) and recorded with a video copy processor (Mitsubishi).

4.2.3.2. Recovery of DNA fragments from low-melting agarose gel

To recover fragments range from 500 pb to 10 kbp from low-melting agarose gel the Gene Clean II kit was used. The agarose gel slice containing the desired DNA fragment was cut out of the agarose gel and transferred into a Falcon tube. 3 volumes of a 3M Na₂J solution was added and incubate at 55°C until the agarose is completely dissolved. To extract DNA from the solution column was used as described in 4.2.2.13.

4.2.4. DNA hybridisation

DNA hybridisation, a process first described by Marmur and Doty (1961), includes the hybridisation of the DNA probe that usually carries some type of labeling that permits localization and quantitation of the other target strand DNA. The standard hybridisation protocol consists of three parts: prehybridization, hybridisation and post hybridisation washing.

4.2.4.1. Dot blot analysis

The application of DNA in solution directly on a hydrophilic cationic nylon membrane is termed a dot blot. This method was used to detect recombinant plasmid DNA after a cloning reaction or to detect recombinant DNA isolated from strains of *S. aurantiaca* after transformation. 5-10 µg chromosomal DNA or 0,5 µg plasmid DNA was dissolved in 2 µl denaturation solution (1M NaOH/50 mM EDTA) followed by denaturation at 98°C for 10 min. DNA samples were spotted on pre-wet nylon membrane and dried at room temperature. The membrane was placed on filter paper saturated with 2xSSC for 5

min, dried and the DNA was fixed by UV irradiation. The membrane was stored at 4°C or directly used for hybridisation.

4.2.4.2. Southern blot analysis (Southern, 1975)

This method is used to transfer restricted DNA from agarose gels to a nylon membrane. Restricted chromosomal or plasmid DNA was separated by agarose gel electrophoresis. It is necessary to reduce the size of the DNA fragments in the gel after electrophoresis to facilitate their transfer to the membrane. Depurination of DNA was accomplished by incubating the gel in 0,25 M HCl for 15 min. Followed by denaturation in high salt buffer 0,5 M NaOH/1,5 M NaCl for 30 min. The gel was neutralized in 1 M Tris/HCl (pH 7, 5)/1,5 M NaCl solution for 30 min. To transfer the DNA from the gel to the nylon membrane vacuum-blotting procedure was used. Transfer was accomplished in transfer buffer (10xSSC) for less than one hour with applied vacuum of 60 mbar using a Vacuum Blotter (Appligene). After transfer DNA was fixed to the membrane by UV irradiation and stored at 4°C or proceed directly to hybridisation.

4.2.4.3. Hybrisation and detection with biotin-labelled probes

Biotin-labelled DNA probes were used for hybridisation with target DNA. Biotin is detected in an immunoassay with streptavidin coupled to alkaline phosphatase (AP). Detection is facilitated using chemiluminiscent AP-substrate CSPD. Enzymatic dephosphorylation of CSPD by AP leads to a light emission at a wavelength of 477 nm which can be recorded on X-ray film.

Prehybridisation of the membrane was performed with 2-4 ml hybridisation solution per 100 cm² of the membrane at 42°C for one hour. Hybridisation solution contains formamide to denaturate DNA. Hybridisation was carried out at 42°C over night with an appropriate volume of hybridisation solution containing the denaturated biotin labelled probe. To remove incorrectly bound probe the membrane was washed once with 2xSSC/0,1%SDS (high stringency) for 30 min and once (low stringency) with 0,1xSSC/0,1SDS for 30 min, at 42 °C with agitation. Detection was performed with the Biotin Detection Kit at the room temperature (Roche Diagnostics). The membrane was rinsed in washing buffer for 5 min and was then incubate in 5xblocking reagent for 30 min. An incubation in 5xblocking solution with 1:5000 streptavidin-AP followed for 30 min. The membrane was rinsed 2x15 min in washing buffer and equilibrated for 5 min in detection buffer. AP substrate CSPD was spread over the membrane and detection of light emission was recorded on a X-ray film.

Hybridisation solution	5xSSC, 5xblocking reagent, 50% formamid, 0,02% SDS, 0,1% N-lauroylsarkosin
10 x Blocking reagent	25 g blocking reagent in 250 ml
Washing buffer	0,1 M maleic acid/0,15 M NaCl (pH 7,5) maleic acid buffer (0,1 M maleic acid/0,15 M NaCl, pH 7,5), 0,3 % Tween 20 (v/v)
Detection buffer	1 mM Tris/HCl, pH 9,5/1 M NaCl

4.2.4.4. Nonradioactive labelling of DNA probes

Nonradioactive labelling of DNA probes was performed with biotin using the Biotin-High Prime kit (Roche). The method is based on the random primed DNA labelling procedure. Hybridisation of oligonucleotides of all possible sequences (random primers) to the denaturated DNA that should be labelled is the first step. The complementary DNA strands is synthesised by Klenow enzyme using the 3`OH termini of the random oligonucleotides as a primer. Modified deoxyribonucleoside-triphosphates labelled with biotin are incorporated into the newly synthesized complementary DNA strand. Biotin-labelled DNA is detected by streptavidin conjugated to the enzyme alkaline phosphatase.

About 1 µg template DNA was denaturated by heating for 10 min to 98°C. 4 µl Biotin-High mixture containing Klenow enzyme, labelling grade, biotin-16-dUTP, dATP, dCTP, dGTP, dTTP in an optimised reaction buffer was added and incubated at 37°C over night. The labelling reaction was stopped by heating at 65°C for 10 min. Long time storage of the labelled probes is possible at -20°C.

4.2.5. Isolation and manipulation of RNA

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

Cells from a log phase culture (about 50 ml) were sedimented by centrifugation at 5000 rpm for 20 min at 4°C. The cell pellet was resuspended in 250 µl of cold sucrose buffer. 125 µl of lysis buffer was added and incubated at 65°C for 90 sec. 250 µl of H₂O-saturated phenol was added and incubated at 65°C for 3 min, followed by incubation at -80°C for 1 min. 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 h. The RNA pellet was washed once with 70% ethanol, air-dried and resuspended in 180 µl of RNA storage buffer. 20 µl of 10xDNase 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 (pH 7) 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 3 M NaAc (pH 7) at -20°C for 3 h. 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/ OD 280 should be 2,0 (1 OD 260 = 40 µl/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	
10xDNase buffer	0,2 mM NaAc (pH 4,5), 0,1 M MgCl ₂ , 0,1 M NaC

4.2.5.2. RNA electrophoresis

RNA electrophoresis was performed on a 1% agarose-formaldehyde gel. One gram of agarose was melted in 72 ml DEPC-treated H₂O and then incubated in 60°C water bath. 18 ml of formaldehyde (37%) and 10 ml of 10xMOPS buffer were added to the agarose suspension. The gel was poured in a hood 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, and then chilled down immediately on ice. 1/10 volume of RNA loading buffer was mixed with the sample before it was loaded on the gel. Electrophoresis was performed in 1 xMOPS buffer at 120 V for 10 min, and then 25 V for about 12 h. The gel was stained with 0,5 µg/ml ethidium bromide solution for 30 min and destained in H₂O for one hour.

10xMOPS buffer	0,2 M MOPS, 80 mM NaAc, 10 mM EDTA (pH 7)
denaturing buffer	20 mM Na-phosphate buffer (pH 6,5), 1 mM EDTA
10xDNase buffer	0,2 M NaAc (pH 4,5), 0,1 M MgCl ₂ , 0,1 M NaCl

4.2.5.3. RT-PCR

The Access RT-PCR kit (Promega), which offers reverse transcription and PCR amplification in a single reaction, was used to detect mRNA expression. First strand cDNA synthesis was performed by incubation at 48°C for 45 min, thereafter the reaction was incubated at 94°C for 2 min to inactivate AMV reverse transcriptase and to denature the RNA/cDNA/primer mixture. Second strand cDNA synthesis and the amplification followed directly through 40 thermal cycle (30 sec at 94°C, 1 min at 54°C and 1 min at 68°C). This was followed by a final extension for 7 min at 68°C. After electrophoresis on an agarose gel, the cDNA was stained with the ethidium bromide for 30 min, and analysed by UV illumination.

4.2.6. Protein purification and analysis

4.2.6.1. Isolation of total protein extract from *S. aurantiaca*

Vegetative *S. aurantiaca* cells (about 15 ml with 2x10⁸ cells/ml) or fruiting bodies that were scratched off the surface of water agar plates after different time of development were sedimented by centrifugation at 5000 rpm for 15 min at 4°C. The pellet was washed in PBS containing 1 mM PMSF. The pellet was stored at -80°C or used immediately for protein purification.

a) Sediments were resuspended in lysis buffer and sonicated in short bursts (8 x 15 sec) at 4°C with 50 % duty cycle in Branson Sonifier cell disruptor B 15. To remove cells debris ultracentrifugation at 30 000 rpm at 15 °C for 30 min was performed. The supernatant was transferred in new tube and the protein concentration was estimated using the Bradford assay. Protein solutions were aliquoted and stored at -80°C. Before electrophoresis 1 volume of the protein solution was mixed with 1 volume of 2xprotein loading buffer

Lysis solution	Urea	7M
	Thiourea	2M

CHAPS	4%
DTT	1%
Tris base	40 mM
Complete (Protease Inhibitor Cocktail, Roche Diagnostics)	

b) Sediments were resuspended in 100 μ l of H₂O and mixed with 100 μ l of 2xprotein loading buffer and heated up to 95°C for 5 min. Followed by sonification in short bursts (8x15 sec) at 4°C with 50% duty cycle. To remove cells debris centrifugation at 13 000 rpm at 4°C for 15 min was performed.

4.2.6.2. Determination of protein concentration (Bradford, 1976)

The protein assay kit (BioRad) was used for the determination of 1-20 μ g protein (concentration less 10 mg/ml) using BSA as a standard protein.

50 μ l of the protein solution was mixed with 1 ml of working reagent Bio-Rad protein assay. The absorbance of the protein solution was measured at 595 nm. Measuring a BSA solution of 0.1- 1 mg/ml at 595 nm was used to set up the standard curve. The concentration of the unknown protein was calculated according to the standard curve.

4.2.6.3. SDS-Polyacrilamide gel electrophoresis (Laemmli, 1970)

Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) is a fast method for quantifying, comparing and characterising proteins. This method separates proteins by their molecular weight. Proteins samples prepared as previously described were separated on 12,5-15% polyacrilamide gels with a 4,5% stacking gel. Electrophoresis was done in 1 x SDS-Tris-Glycine buffer at 25mA.

Stacking gel: Tris-HCl (pH 6,8) 125 mM; Acrylamide:bis-acrylamide (30:0,8) 4,5%; SDS 0,1%; APS 0,4%; TEMED 0,4%.

Separating gel: Tris-HCl (pH 8,8) 375 mM; Acrylamide:bis-acrylamide (30:0,8) 8-15%; SDS 0,1%; APS 0,4%; TEMED 0,25%.

2xProtein loading buffer: 125 mM Tris-HCl (pH 6,8); 4% SDS; 10% β -mercaptoethanol; 10% glycerol; 0,02% Bromophenol blue;

10xSDS-Tris-Glycin buffer 330 mM Tris-HCl (pH 6,8); 1,90 M glycine; 1% SDS

4.2.6.4. Coomassie blue staining of gels

After electrophoresis the polyacrylamide gel was stained with Coomassie Brilliant Blue R250 for about 30 min at room temperature with gentle shaking. Then the gel was destained for 1-2 hours at room temperature with gentle shaking in destaining solution. The destaining solution was changed at least twice. Using the gel for immunoblotting the transfer was performed immediately without staining.

Staining solution 45% methanol; 10% acetic acid; 0,2% Coomassie brilliant blue R250.

Destaining solution 5% methanol; 4,2% glacial acetic acid

4.2.6.5.2.2. Detection of the AP conjugated secondary antibody

After incubation with AP-labelled secondary antibody the membrane was incubated in AP detection buffer with 0,67% NBT solution and 0,33% BCIP solution until the desired signals were obtained. The reaction was stopped by transferring the membrane into a 50 mM EDTA solution (pH 8).

AP buffer 0,1M Tris-HCl (pH 9,5); 5 mM EDTA; 5 mM MgCl₂

4.2.6.6. Anti-peptide antibodies

4.2.6.6.1. Synthesis of the peptides

In order to produce a serum raised against specific epitops of a certain protein two peptides were synthesised using the solid-phase techniques (Merrifield, 1963) in the Peptide Specialty Laboratories GmbH. Synthetic peptides were purified by HPLC and stored at -20°C.

4.2.6.6.2. Coupling of the peptides to a carrier protein

Small molecules like peptides, although able to interact with products of an immune response, cannot stimulate a response. Peptides can be immunogenic only when they are exposed on the surface of a larger carrier protein. A keyhole limpet hemocyanin (KLH) was used as carrier protein due to its large molecular mass, strong immunogenicity and many available lysines.

KLH was preactivated with a heterobifunctional cross-linker (sulfo-SMCC). Conjugation of the peptide sulfhydryl group of last Cys and maleimide activated KLH was performed using the kit Inject Maleimide Activated Carrier Proteins (Pierce).

4.2.6.6.3. Immunisation

Peptide mixture was used for immunisation of two rabbits following the standard immunisation protocol for three months (Peptide Specialty Laboratories GmbH). Elsa test using the peptides coupled to OVA as controls evaluated the serum titer.

The final serum was divided into smaller fractions and stored at -80°C for long time storage. An aliquot used frequently was stored at 4°C.

4.2.6.6.4. Purification of serum on peptide columns

Each peptide was coupled to the Sulfo-Link coupling gel necessary for further serum purification. The columns were made in Peptide Specialty Laboratories GmbH.

Before purification 1 ml of the column matrix was equilibrated with PBS buffer. 5 ml of serum was diluted with an equal volume of PBS buffer, mixed with 1 ml of the column matrix and incubated over night at 4°C with slowly rotation. The suspension was transferred to the column. The matrix settled in the column and the flow through

was collected. The column was washed twice with 5 ml PBS buffer and then again three times with 5 ml 10 mM Na-phosphate (pH 6,8). All washing steps were performed at 4°C. Antibodies were eluted from the column with 10x0,5 ml 0,1 M glycine-buffer (pH 2,82) in Eppendorf tubes containing 50 μ l 1 M Tris HCl (pH 8). The protein concentration of each eluted fraction was estimated by Bradford assay. Purified serum was divided into 100 μ l aliquots and the equal volume of 98% glycerol and 0,05% (v/v) Na-azide was added. The aliquots were stored at -80°C.

The column was regenerated by washing with 10 ml 10 mM Na-phosphate buffer (pH 6,8), then two times with 10 ml PBS containing 1 M NaCl and finally two times with 10 ml PBS containing 0,05% Na-azide. The column can be stored at 4 °C.

4.2.6.7. Antibody against fusion protein

4.2.6.7.1. Preparation of *E. coli* cell lysate

1 litre of the induced bacterial cultures was centrifuged and the pellet was resuspended in lysis buffer A, 5 ml per gram wet weight (6M guanidine hydrochloride; 0,1 M NaH₂PO₄; 0,01 M Tris-HCl (pH 8)). The cell suspension was stirred over night at 4°C and centrifuged at 16 000 rpm for 20-40 min at 4 °C to remove the cells debris. Supernatant was sonicated 8 x 15 sec with duty cycle 50% and centrifuged at 16000 rpm for 40 min at 4 °C. Then the supernatant was filtrated through a 0,45 μ m sterile filter and once through a 0,22 μ m filter. The protein solution was stored for several days at 4°C or used immediately for purification.

4.2.6.7.2. Purification of fusion protein on Ni-column

HiTrap chelating HP 5 ml column (Amersham pharmacia) packed with Chelating Sepharose High Performance was used for purification. The separation was achieved in a ÄKTA liquid chromatography system. The column was charged with 0,1 M NiSO₄ solution and washed with about 15 ml distilled water. After column preparation an equilibration with 5-10 column volumes buffer B with a flow rate of 5ml/min followed. The sample was loaded on the column with a flow rate of 1 ml/min and washed with 5-10 column volumes of buffer B until absorbance at 280 nm was below 0,01. Washing with 5-10 column volumes of buffer C was performed to remove proteins that bind non-specifically to the resin. Fusion protein was eluted with buffer D using the imidazole linear gradient.

After purification the metal ions were stripped from resin by washing with 5 column volumes buffer containing 0,05 M EDTA. This was followed by washing with 5-10 column volumes of distilled water. The column was stored in 20% ethanol.

All buffers were filtrated through a 0,45 μ m filter before use.

Lysis buffer B	8M urea; 0,1 M NaH ₂ PO ₄ ; 0,01 M Tris-HCl (pH 8).
Washing buffer C	8 M urea; 0,1 M NaH ₂ PO ₄ ; 0,01 M Tris-HCl (pH 6,3)
Elution buffer D	8M urea; 0,1 M NaH ₂ PO ₄ ; 1 M imidazol; 0,01 M Tris-HCl (pH 6,3)

4.2.6.7.3. Immunisation with fusion protein

Two rabbits were immunised with the fusion protein following standard three months procedure in the laboratories of SEQ -Lab (Göttingen).

4.2.6.7.4. Concentrating protein solutions

The protein solutions were concentrated *via* dialyses. Dialysis is typically used for changing the buffer solution of a protein but is also a method for concentrating protein solutions by dialysing against a hygroscopic environment (Sephadex). The protein solution is within a membrane whose pore size prevents the proteins from escaping.

Dialysing tubes were cleaned from chemical contaminants from the manufacturing process by boiling for 30 min in 10 mM NaHCO₃/1 mM EDTA. After that the tubes were washed extensively in distilled water and stored at 4°C in 20% ethanol to prevent microbial contamination.

The protein solution was placed in dialysing tubes and embedded in the Sephadex G-25 resin at room temperature. The resin was changed every half hour to achieve concentration of the protein sample in a shorter period of time.

4.2.6.8. Determination of β -galactosidase activity (Ruan et al., 1993)

Protein was isolated from vegetative cells or from fruiting bodies/aggregates after different times after inducing starvation. Cells were pelleted by centrifugation at 5000 rpm for 20 min at 4°C. Pellets were washed once in MOPS buffer and resuspended in 150 μ l of the same buffer. A small spoon of glass beads (0,1 mm) was added to each sample and cells were broken by sonification at 4°C with the Branson sonifier B-15 with shorts bursts (15 sec), 50 % duty cycle. After centrifugation at 13 000 rpm to remove glass beads the protein solution was centrifugated at 45 000 rpm for 1 h at 4 °C. The protein concentration was determined as previously escribed. The protein extracts were stored at -80°C.

10 μ g of the protein sample was diluted in 100 μ l degassed MOPS buffer and mixed with 300 μ l buffer A. Following incubation at 37°C for 30 min the reaction was stopped by addition of 3 ml 0,1 M degassed Glycin buffer. Buffer A contains the fluorescent substrate 4-MUG (4-Methylumbelliferyl- β -D-Galaktopyransid) The substrate can be cleaved by β -galactosidase releasing the fluorescent methylumbelliferon that can be 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 (pH 7) Na-phosphate; 0,1 M NaCl; 1 mM MgCl₂; 10 μ g/300 ml
 4-MUG; 0,1% BSA

MOPS buffer 50 mM MOPS; 0,01 M MgCl₂

4.3. Software

Platform	Software	Task
Apple Macintosh	DNA Strider	Sequence editor
	Laser gene	Sequence editor Mapping of restriction enzyme sites Multiple alignments of sequences Protein analysis
	Adobe Photoshop	Image processing
www searches	BLAST	Protein and nucleotide similarity
		Protein second structure analysis

V. Summary

Cell-cell interaction is a prerequisite for multi-cellular development and cellular differentiation of the Gram-negative bacterium *Stigmatella aurantiaca*. For the elucidation of the temporal and spatial coordination of the physiology and motility of the cells during development, isolation and characterization of molecules involved in cell-cell signalling is needed.

The best studied intercellular signal of *Myxococcus xanthus* is the C signal, which is the gene product of the *csgA* gene. Using the *M. xanthus csgA* gene as probe a homologous gene was previously isolated from *S. aurantiaca* (Butterfass, 1992). Inactivation of the gene by insertional mutagenesis caused alterations in *S. aurantiaca* fruiting.

A *Xho*I fragment harbouring the *csgA* gene and flanking regions was isolated. Sequence analysis revealed additional putative start codons located upstream of the proposed *csgA* GTG translational start. Based on the homology data of the *M. xanthus csgA* gene the best reading frame indicates that the *csgA* translational start ATG codon is located 189 bp upstream of GTG. It specifies a protein of 236 amino acids with an estimated molecular mass of 26 kDa. The CsgA protein appears to be a member of the SRD family. The putative catalytic site (Ser139, Tyr158 and Lys162) is highly conserved in CsgA, as well as the putative coenzyme binding domain of the protein. Further, a new ORF, the protoporphyrinogene oxidase gene, was found upstream of *csgA* in the opposite orientation, 242 bp apart from *csgA*. The deduced amino acid sequence of this ORF has significant similarity with protoporphyrinogene oxidase from *M. xanthus*. An unknown ORF, *orf2*, was found upstream of *csgA* in same orientation and 251 bp apart

from *csgA*, which encodes a polypeptide of 247 amino acids. No similarity was found between the deduced amino acid sequence of *orf2* product and known proteins. An ORF, *fprA*, was found downstream of *csgA* in the opposite orientation. It overlaps with *csgA* (55 bp). The *fprA* gene specifies a protein of 223 amino acids with significant similarity to the flavin associated protein from *M. xanthus*.

Due to the strong evidences supporting the role of CsgA in *M. xanthus* intercellular signalling and the close phylogenetic relationship between *Stigmatella* and *Myxococcus* it was speculated that the CsgA protein plays a role in communication between *S. aurantiaca* cells during development. A *S. aurantiaca csgA* insertion-mutant was constructed. CsgA mutant cells show an altered developmental behaviour as compared with wild type cells. The motility behaviour of the cells during development was changed and their ability to stay more closely together in the early stages of development. Inactivation of the *csgA* gene completely abolished rippling of the cells. This indicates the crucial role of the CsgA protein in regulating this rhythmic behaviour.

S. aurantiaca csgA mutant cells do not produce CsgA but they are able to respond to it when mixed with wild type cells. Mixing the cells of the *S.aurantiaca csgA* mutant with those of a mutant that expresses the green fluorescence protein, resulted in wild-type fruiting body with an intermediate colour (orange / green).

The *csgA* promoter seems to be very weak. Promoter activity of *csgA* was studied using a promoterless \square *trpA-lacZ* gene as reporter gene. \square -galactosidase activity was very low and increased weakly at the beginning of the starvation induced development. A 0.6 kbp putative promoter region is sufficient for *csgA* expression.

The concentration of the CsgA protein is low. This is a consequence of weak expression of the *csgA* gene. CsgA probably acts in the pM range as a signal *per se* or has an enzymatic function to convert a substrate into the signal molecule.

VI. References

- Burchard, R.P. 1984. Girdling motility and taxes, p. 139-161. In E. Rosenberg (ed.), *Myxobacteria: development and cell interactions*. Springer-Verlag, New York.
- 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.L., Kalman, L.V. et al. (1994). "The *dsg* gene of *Myxococcus xanthus* encodes a protein similar to translation initiation factor IF3." J Bacteriol **176**: 1427-33.
- Buterfass, H.J. Diploma thesis. Ruprecht-Karls-Universität, Heidelberg, Germany.
- Crawford, E. W., Jr. and L. J. Shimkets (2000). "The *Myxococcus xanthus* *socE* and *csgA* genes are regulated by the stringent response." Mol Microbiol **37**(4): 788-99.
- Crawford, E. W., Jr. and L. J. Shimkets (2000). "The stringent response in *Myxococcus xanthus* is regulated by SocE and the CsgA C-signaling protein." Genes Dev **14**(4): 483-92.
- Dailey, H. A. and T. A. Dailey (1996). "Protoporphyrinogen oxidase of *Myxococcus xanthus*. Expression, purification, and characterization of the cloned enzyme." J Biol Chem **271**(15): 8714-8.
- Downard, J. and D. Toal (1995). "Branched-chain fatty acids: the case for a novel form of cell-cell signalling during *Myxococcus xanthus* development." Mol Microbiol **16**(2): 171-5.
- Dworkin, M. (1963). "Nutritional regulation of morphogenesis in *Myxococcus xanthus*". J Bacteriol **86**:67-72.
- Dworkin, M. (1985). *Developmental biology of the bacteria*. The Benjamin/Cummings Publishing Company, Inc, California.
- Dworkin, M. (1994). "Increases in the intercellular concentration of glycerol during development in *Myxococcus xanthus* S. Courtney Frasch [corrected and republished in FEMS Microbiol Lett. 194 Oct 1; 122(3): 321-5]. FEMS Microbiol. Lett, 120: 396-373.
- Ellehaug, E., M. Norregaard-Madsen, et al. (1998). "The FruA signal transduction protein provides a checkpoint for the temporal co-ordination of intercellular signals in *Myxococcus xanthus* development." Mol Microbiol **30**(4): 807-17.
- Engbrecht, J., K. Nealson, et al. (1983). "Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*." Cell **32**(3): 773-81.
- Ensor, C. M. and H. H. Tai (1991). "Site-directed mutagenesis of the conserved tyrosine 151 of human placental NAD(+)-dependent 15-hydroxyprostaglandin dehydrogenase yields a catalytically inactive enzyme." Biochem Biophys Res Commun **176**(2): 840-5.
- Errington, J. (1993). "Bacillus subtilis sporulation: regulation of gene expression and control of morphogenesis." Microbiol Rev **57**(1): 1-33.
- Flavier, A. B., L. M. Ganova-Raeva, et al. (1997). "Hierarchical autoinduction in *Ralstonia solanacearum*: control of acyl-homoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester." J Bacteriol **179**(22): 7089-97.

- Gamard, P., F. Sauriol, et al. (1997). "Novel butyrolactones with antifungal activity produced by *Pseudomonas aureofaciens* strain 63-28." J Antibiot (Tokyo) **50**(9): 742-9.
- Gerth, K., N. Bedorf, et al. (1996). "Epothilons A and B: antifungal and cytotoxic compounds from *Sorangium cellulosum* (Myxobacteria). Production, physico-chemical and biological properties." J Antibiot (Tokyo) **49**(6): 560-3.
- Gerth, K., N. Bedorf, et al. (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**(1): 23-31.
- Gerth, K., H. Irschik, et al. (1980). "Myxothiazol, an antibiotic from *Myxococcus fulvus* (myxobacterales). I. Cultivation, isolation, physico-chemical and biological properties." J Antibiot (Tokyo) **33**(12): 1474-9.
- Gerth, K., R. Metzger, et al. (1993). "Induction of the myxospore in *Stigmatella aurantiaca* (myxobacteria)- Inducers and inhibitors of myxospore formation, and mutants with a changed sporulation behaviour." J. Gen. Microbiol. **139**: 865-871.
- Gill, R. E. and M. G. Cull (1986). "Control of developmental gene expression by cell-to-cell interactions in *Myxococcus xanthus*." J Bacteriol **168**(1): 341-7.
- Gill, R. E., M. Karlok, et al. (1993). "*Myxococcus xanthus* encodes an ATP-dependent protease which is required for developmental gene transcription and intercellular signaling." J Bacteriol **175**: 4538-44.
- Gronewold, T. M. and D. Kaiser (2001). "The *act* operon controls the level and time of C-signal production for *Myxococcus xanthus* development." Mol Microbiol **40**(3): 744-56.
- Hagen, D. C., A. P. Bretscher, et al. (1978). "Synergism between morphogenetic mutants of *Myxococcus xanthus*." Dev Biol **64**(2): 284-96.
- Hagen, T. J. and L. J. Shimkets (1990). "Nucleotide sequence and transcriptional products of the *csg* locus of *Myxococcus xanthus*." J Bacteriol **172**(1): 15-23.
- Hartzell, P. and D. Kaiser (1991). "Function of MglA, a 22-kilodalton protein essential for gliding in *Myxococcus xanthus*." J Bacteriol **173**(23): 7615-24.
- 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**(18): 9881-6.
- Heidelbach, M., H. Skladny, et al. (1993). "Purification and characterization of SP21, a development-specific protein of the myxobacterium *Stigmatella aurantiaca*." J Bacteriol **175**(3): 905-8.
- Hoang, T. T., S. A. Sullivan, et al. (2002). "Beta-ketoacyl acyl carrier protein reductase (FabG) activity of the fatty acid biosynthetic pathway is a determining factor of 3-oxo- homoserine lactone acyl chain lengths." Microbiology **148**(Pt 12): 3849-56.
- Hodgkin, J., and D. Kaiser (1979). "Genetics of gliding motility in *Myxococcus xanthus* (Myxobacterales): two gene systems control movements". Mol. Gen. Genet. (172): 177-191.
- Hodgkin, J., and D. Kaiser (1979). "Genetics of gliding motility in *Myxococcus xanthus* (Myxobacterales): genes controlling movements of single cell". Mol. Gen. Genet. (171): 167-176.
- Hoiczyk, E. and W. Baumeister (1998). "The junctional pore complex, a prokaryotic secretion organelle, is the molecular motor underlying gliding motility in cyanobacteria." Curr Biol **8**(21): 1161-8.

- Horinouchi, S. and T. Beppu (1992). "Regulation of secondary metabolism and cell differentiation in *Streptomyces*: A-factor as a microbial hormone and the AfsR protein as a component of a two-component regulatory system." Gene **115**(1-2): 167-72.
- Hull, W. E., A. Berkessel, et al. (1998). "Structure elucidation and chemical synthesis of stigmolone, a novel type of prokaryotic pheromone." Proc Natl Acad Sci U S A **95**(19): 11268-73.
- Janssen, G. R. and M. Dworkin (1985). "Cell-cell interactions in developmental lysis of *Myxococcus xanthus*." Dev Biol **112**(1): 194-202.
- Jelsbak, L. and L. Sogaard-Andersen (1999). "The cell surface-associated intercellular C-signal induces behavioral changes in individual *Myxococcus xanthus* cells during fruiting body morphogenesis." Proc Natl Acad Sci U S A **96**(9): 5031-6.
- Ji, G., R. C. Beavis, et al. (1995). "Cell density control of staphylococcal virulence mediated by an octapeptide pheromone." Proc Natl Acad Sci U S A **92**(26): 12055-9.
- Kaiser, D. (1979). "Social gliding is correlated with the presence of pili in *Myxococcus xanthus*." Proc Natl Acad Sci U S A **76**(11): 5952-6.
- Kaiser, D. (2000). "Bacterial motility: how do pili pull?" Curr Biol **10**(21): R777-80.
- Kaplan, H. B. and E. P. Greenberg (1985). "Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system." J Bacteriol **163**(3): 1210-4.
- Kim, S. K. and D. Kaiser (1990). "Purification and properties of *Myxococcus xanthus* C-factor, an intercellular signaling protein." Proc Natl Acad Sci U S A **87**(10): 3635-9.
- Komano, T., S. Inouye, et al. (1980). "Patterns of protein production in *Myxococcus xanthus* during spore formation induced by glycerol, dimethyl sulfoxide, and phenethyl alcohol." J Bacteriol **144**(3): 1076-82.
- Korolev, E. V., A. V. Nikonov, et al. (1994). "Tubular structures of *Mycoplasma gallisepticum* and their possible participation in cell motility." Microbiology **140** (Pt 3): 671-81.
- Kroos, L. and D. Kaiser (1987). "Expression of many developmentally regulated genes in *Myxococcus* depends on a sequence of cell interactions." Genes Dev **1**(8): 840-54.
- Kruse, T., S. Lobedan, et al. (2001). "C-signal: a cell surface-associated morphogen that induces and co-ordinates multicellular fruiting body morphogenesis and sporulation in *Myxococcus xanthus*." Mol Microbiol **40**(1): 156-68.
- Kunze, B., T. Kemmer, et al. (1984). "Stigmatellin, a new antibiotic from *Stigmatella aurantiaca* (Myxobacterales). I. Production, physico-chemical and biological properties." J Antibiot (Tokyo) **37**(5): 454-61.
- Kuspa, A. and D. Kaiser (1989). "Genes required for developmental signalling in *Myxococcus xanthus*: three *asg* loci." J Bacteriol **171**(5): 2762-72.
- Kuspa, A., L. Plamann, et al. (1992). "Identification of heat-stable A-factor from *Myxococcus xanthus*." J Bacteriol **174**(10): 3319-26.
- Kuspa, A., L. Plamann, et al. (1992). "A-signalling and the cell density requirement for *Myxococcus xanthus* development." J Bacteriol **174**(22): 7360-9.
- LaRossa, R., J. Kuner, et al. (1983). "Developmental cell interactions of *Myxococcus xanthus*: analysis of mutants." J Bacteriol **153**(3): 1394-404.

- Lee, B. U., K. Lee, et al. (1995). "A tactile sensory system of *Myxococcus xanthus* involves an extracellular NAD(P)(+)-containing protein." Genes Dev **9**(23): 2964-73.
- Li, S., B. U. Lee, et al. (1992). "csgA expression entrains *Myxococcus xanthus* development." Genes Dev **6**(3): 401-10.
- Lunsdorf, H. and H. U. Schairer (2001). "Frozen motion of gliding bacteria outlines inherent features of the motility apparatus." Microbiology **147**(Pt 4): 939-47.
- MacNeil, S. D., F. Calara, et al. (1994). "New clusters of genes required for gliding motility in *Myxococcus xanthus*." Mol Microbiol **14**(1): 61-71.
- Merz, A. J., M. So, et al. (2000). "Pilus retraction powers bacterial twitching motility." Nature **407**(6800): 98-102.
- Müller, S. 2002. Ph.D. thesis. Ruprecht-Karls-Universität, Heidelberg, Germany.
- Neumann, B., A. Pospiech, et al. (1992). "Size and stability of the genomes of the myxobacteria *Stigmatella aurantiaca* and *Stigmatella erecta*." J Bacteriol **174**(19): 6307-10.
- Neumann, B., A. Pospiech, et al. (1993). "A physical and genetic map of the *Stigmatella aurantiaca* DW4/3.1 chromosome." Mol Microbiol **10**(5): 1087-99.
- O'Connor, K. A. and D. R. Zusman (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**(4): 839-50.
- Obeid, J. and P. C. White (1992). "Tyr-179 and Lys-183 are essential for enzymatic activity of 11 beta- hydroxysteroid dehydrogenase." Biochem Biophys Res Commun **188**(1): 222-7.
- Persson, B., M. Krook, et al. (1991). "Characteristics of short-chain alcohol dehydrogenases and related enzymes." Eur. J. Biochem. **200**: 537-43.
- Pestova, E. V., L. S. Havarstein, et al. (1996). "Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system." Mol Microbiol **21**(4): 853-62.
- Pfister, D. H. (1984). "Roland Taxter: mycologist and entomologist." Fungus-Insect Relationships: Perspectives in Ecology and Evolution.: 211-221.
- Piggot, P. J. and J. G. Coote (1976). "Genetic aspects of bacterial endospore formation." Bacteriol Rev **40**(4): 908-62.
- Plaga, W., I. Stamm, et al. (1998). "Intercellular signaling in *Stigmatella aurantiaca*: purification and characterization of stigmolone, a myxobacterial pheromone." Proc Natl Acad Sci U S A **95**(19): 11263-7.
- Plamann, L., A. Kuspa, et al. (1992). "Proteins that rescue A-signal-defective mutants of *Myxococcus xanthus*." J Bacteriol **174**(10): 3311-8.
- Pollack, J. S. and M. Singer (2001). "SdeK, a histidine kinase required for *Myxococcus xanthus* development." J Bacteriol **183**(12): 3589-96.
- Pospiech, A., B. Neumann et al. (1993). "Detection of developmentally regulated gens of the myxobacterium *Stigmatella aurantiaca* with the transposon Tn5lacZ". Arch. Microbiolo. **159**: 201-206.
- Qualls, G. T., K. Stephens, et al. (1978). "Light-stimulated morphogenesis in the fruiting myxobacterium *Stigmatella aurantiaca*." Science **201**(4354): 444-5.
- Qualls, G. T., K. Stephens, et al. (1978). "Morphogenetic movements and multicellular development in the fruiting Myxobacterium, *Stigmatella aurantiaca*." Dev Biol **66**(1): 270-4.

- Raman, R. K., Y. Hashimoto, et al. (1976). "Differentiation for aggregation in the cellular slime moulds: the emergence of autonomously signalling cells in *Dictyostelium discoideum*." J Cell Sci **21**(2): 243-59.
- Reichenbach, H. (1986). "The myxobacteria: common organisms with uncommon behaviour." Microbiol Sci **3**(9): 268-74.
- Reichenbach, H. and M. Dworkin (1970). "Induction of myxospore formation in *Stigmatella aurantiaca* (Myxobacterales) by monovalent cations." J Bacteriol **101**(1): 325-6.
- Rhie, H. G. and L. J. Shimkets (1989). "Developmental bypass suppression of *Myxococcus xanthus* *csgA* mutations." J Bacteriol **171**(6): 3268-76.
- Rodriguez-Soto, J. P. and D. Kaiser (1997). "The *tgl* gene: social motility and stimulation in *Myxococcus xanthus*." J Bacteriol **179**(13): 4361-71.
- Ruan, K. H., R. J. Kulmacz, et al. (1993). "Highly sensitive fluorimetric enzyme immunoassay for prostaglandin H synthase solubilized from cultured cells." J Immunol Methods **162**(1): 23-30.
- Thöny-Meyer, L. and Kaiser D. (1993). "devRS, an auto regulated and essential genetic locus for fruiting body development in *Myxococcus xanthus*." J. Bacteriol. **175**:7450-62.
- Sager, B. and D. Kaiser (1994). "Intercellular C-signaling and the traveling waves of *Myxococcus*." Genes Dev **8**(23): 2793-804.
- Schairer, H.U. (1993). "*Stigmatella aurantiaca*, an organism for studying the genetic determination of morphogenesis". In Dworkin, M. and Kaiser, D. (ed.), *Myxobacteria II*. American Society of Microbiology, Washington, D.C. p. 333-346.
- Shimkets, L. J. (1989). "The role of the cell surface in social and adventurous behaviour of myxobacteria." Mol Microbiol **3**(9): 1295-9.
- Shimkets, L. J. (1990). "The *Myxococcus xanthus* FprA protein causes increased flavin biosynthesis in *Escherichia coli*." J Bacteriol **172**(1): 24-30.
- Shimkets, L. J. (1999). "Intercellular signaling during fruiting-body development of *Myxococcus xanthus*." Annu Rev Microbiol **53**: 525-49.
- Shimkets, L. J. and S. J. Asher (1988). "Use of recombination techniques to examine the structure of the *csg* locus of *Myxococcus xanthus*." Mol Gen Genet **211**(1): 63-71.
- Shimkets, L. J. and M. Dworkin (1981). "Excreted adenosine is a cell density signal for the initiation of fruiting body formation in *Myxococcus xanthus*." Dev Biol **84**(1): 51-60.
- Shimkets, L. J., R. E. Gill (1983). "Developmental cell interactions in *Myxococcus xanthus* and the *spoC* locus." Proc. Natl. Acad. Sci. USA **80**: 1406-10.
- Shimkets, L. J. and D. Kaiser (1982). "Induction of coordinated movement of *Myxococcus xanthus* cells." J Bacteriol **152**(1): 451-61.
- Shimkets, L. J. and H. Rafiee (1990). "CsgA, an extracellular protein essential for *Myxococcus xanthus* development." J Bacteriol **172**(9): 5299-306.
- Silakowski, B., H. Ehret, et al. (1998). "*fbfB*, a gene encoding a putative galactose oxidase, is involved in *Stigmatella aurantiaca* fruiting body formation." J Bacteriol **180**(5): 1241-7.
- Silakowski, B., A. Pospiech, et al. (1996). "*Stigmatella aurantiaca* fruiting body formation is dependent on the *fbfA* gene encoding a polypeptide homologous to chitin synthases." J Bacteriol **178**(23): 6706-13.

- Silakowski, B., H. U. Schairer, et al. (1999). "New lessons for combinatorial biosynthesis from myxobacteria. The myxothiazol biosynthetic gene cluster of *Stigmatella aurantiaca* DW4/3-1." J Biol Chem **274**(52): 37391-9.
- She W., Zusman D.R. (1993). "The two motility system of *Myxococcus xanthus* show differnt selective advantages on various surfaces." Proc Natl Acad Sci U S A **90**: 3378-82.
- Skladny, H., M. Heidelberg, et al. (1994). "Cloning and characterization of the gene encoding the major sigma factor of *Stigmatella aurantiaca*." Gene **143**(1): 123-7.
- Sogaard-Andersen, L. and D. Kaiser (1996). "C factor, a cell-surface-associated intercellular signaling protein, stimulates the cytoplasmic Frz signal transduction system in *Myxococcus xanthus*." Proc Natl Acad Sci U S A **93**(7): 2675-9.
- Spormann, A. M. (1999). "Gliding motility in bacteria: insights from studies of *Myxococcus xanthus*." Microbiol Mol Biol Rev **63**(3): 621-41.
- Stephens, K., P. Hartzell, et al. (1989). "Gliding motility in *Myxococcus xanthus*: mgl locus, RNA, and predicted protein products." J Bacteriol **171**(2): 819-30.
- Sun, H., D. R. Zusman, et al. (2000). "Type IV pilus of *Myxococcus xanthus* is a motility apparatus controlled by the frz chemosensory system." Curr Biol **10**(18): 1143-6.
- Telford, G., D. Wheeler, et al. (1998). "The *Pseudomonas aeruginosa* quorum-sensing signal molecule N-(3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity." Infect Immun **66**(1): 36-42.
- Thaxter, R. (1982). "Contributions from the Cryptogamic Laboratory of Harvard University. XVIII. On the *Myxobacteriaceae*, a new order of *Schizomycetes*." Bot.Gaz. **14**: 389-406.
- Toal, D. R., S. W. Clifton, et al. (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**(2): 177-89.
- Wall, D., P. E. Kolenbrander, et al. (1999). "The *Myxococcus xanthus* *pilQ* (*sglA*) gene encodes a secretin homolog required for type IV pilus biogenesis, social motility, and development." J Bacteriol **181**(1): 24-33.
- Ward M.J., Zusman, D. R., (1997). "Regulation of direct motility in *Myxococcus xanthus*." Mol Microbiol **24**: 885-893.
- Ward M.J., Zusman, D. R., (1999). " Motility in *Myxococcus xanthus* and its role in developmental agreggation." Curr. Opin.Microbiol. **2**: 624-629.
- White, D. J. and P. L. Hartzell (2000). "AglU, a protein required for gliding motility and spore maturation of *Myxococcus xanthus*, is related to WD-repeat proteins." Mol Microbiol **36**(3): 662-78.
- Wirth, R., A. Muscholl, et al. (1996). "The role of pheromones in bacterial interactions." Trends Microbiol **4**(3): 96-103.
- Wu, S. S., J. Wu, et al. (1998). "The *pilH* gene encodes an ABC transporter homologue required for type IV pilus biogenesis and social gliding motility in *Myxococcus xanthus*." Mol Microbiol **29**(5): 1249-61.
- Wu, S. S., J. Wu, et al. (1997). "The *Myxococcus xanthus* *pilT* locus is required for social gliding motility although pili are still produced." Mol Microbiol **23**(1): 109-21.
- Zusman, D. R. (1980). "Genetic aproaches to the study of develoment in the Myxobacteria. In T. Leighton and H. Parish (ed.), *Molecular gebnetics of development*, p. 41-78. Academic Press, New York.

- Zusman, D. R. (1982). "Friszy" mutants: a new class of aggregation-defective developmental mutants of *Myxococcus xanthus*." J Bacteriol **150**(3): 1430-7.

VII. Appendices

Appendix 1. Abbreviations

<i>B. subtilis</i>	<i>Bacillus subtilis</i>
<i>D. discoideum</i>	<i>Dictyostelium discoideum</i>
<i>S. aurantiaca</i>	<i>Stigmatella aurantiaca</i>
<i>M. xanthus</i>	<i>Myxococcus xanthus</i>
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>V. fisheri</i>	<i>Vibrio fisheri</i>
<i>E. coli</i>	<i>Escherichia coli</i>
amp ^r	ampicillin resistance
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Fig.	figure
β-gal.	β-galactosidase
HPLC	high performance liquid chromatography
hr	hour
IgG	immunoglobulin G
km ^r	kanamycin resistance
kbp	kilobasepair
kDa	kilodalton
M	molar
mg	miligram
min	minute
mM	milimolar
mRNA	messenger ribonucleic acid
nts	nucleotides
OD	optical density
ORF	open reading frame
PAGE	poly-acrylamide gel electrophoresis
RBS	ribosome binding site
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
str ^r	streptomycin resistance
tet ^r	tetracyclin resistance
μg	microgram
μl	microlitre
nM	nanomolar

v/v	volume to volume
w/v	weight to volume
°C	degree Celsius

Appendix 2. *Xho*I DNA fragment – ORFs

GGCCTCGAGGTGGCCACGGTGTATTGCGGAATTCCTCGGCCAGCGGACGGCTCCGTGAAGTCCGGGCTCGCCGTCA 80
 G R P P G R H I A S N G E A L P R G G H L G P E G D
 CCCCggccATCTCGCGAGCTCCTGTGGGCCAGTGCCGCCAGGGCTTCTTCGTTCAGCCCCACGAGGTCTGGCTCCGC 160
 G R G D R A A G A P G T G G P S R R E A G R P R A E A
 GCGCCCCCATCAGGCACGTGTAGAGGACACGGCCGCCCTCCGCGCAAAGGGGAACACCGTGGAGACGTGGATGACGCC 240
 R G G D P V H L P C P R G G R S L P V G H L R P H R G
 CAGCAGTTGCCGCTGCTCCTGGCCCCGAACGAGGAAGCCAAAGCCATCGGGCGGGGGCGTCTTTCCTGGGGCAAAGCCCA 320
 A T A A A G P G S R P L W L W R A P A D K R P C L G
 GGTGCACCACGGCGATGGGGGCGTAGGGAATGCCCCTCAGGTGCCCCGCGAGTGACGGATCCAAGGGGGCGAGCAGCTCC 400
 P A G R R H P R L S H G E P A G R T V S G L P R A A G
 M P F R C P A S D G S K G A S S S
 GCGGACACATGGGCCGGTGAGGTCAACACCACCTGGGAGGCCTCCAGCTCCGCCTGCTGTCCCCCGCTCACGCACCGAGA 480
 R V C P G T L D V G G P L G G A G G A T G R E R V S L
 A D T W A G E V N T T W E A S S S A C C P P L T H R E
 GCGCCATCCGTTCTGGCTTCGCGCGAGCCCTTCCACCGCCGCGCCGTGCGGGCCGCGGGGCCGAGTTGCTCGCGCCA 560
 R W G N Q S R A L G E V A A G T R A A P G S N S A G
 A P S V L A S R E P F H R R A R A G R G A R V A R A
 GGGCTTCCACCAGCGTGCCCAAGCCTCCCTCGAACGTGCACATGGCCCCCTCAGCTTCGTGCCCGCGGGGGCCGGCGC 640
 P S E V L T G L G G E F T C M A G K L K T G A P A P A
 R A F H Q R A Q A S L E R A H G P L Q L R A R G G R R
 GCGCTGCCCTCCCTGGGTTCTCACGGCGCCGAGCAGGAGGCTGCGGTGCTCGCGCTCCAGCTGCTTCAGCGTGGGAAAGG 720
 R Q R G Q T R V A G L L L S R H E R E L Q K L T P F A
 A L P P L G S H G A E Q E A A V L A L Q L L Q R G K G
 CGGCCCTCCGCGCTCAGCGCCTCCACGTCCCCCGGTAGGTGCCCGTCTGCATCGCGTCCAGCAGCACCGACGTGGCGCGC 800
 A E A S L A E V D G A Y T G T Q M A D L L V S T A R
 G L R A Q R L H V P R V G A R L H R V Q Q H R R G A
 GCGCCACGTGCCTCCGCCGAAGTCACCGAGGGATTGCTCCTGGCTGGCGGCGCGGGCCCGTGAACAACCTCCGCGAG 880
 A G V H R R G F D G L S E D Q G P P A R G T F L E A L
 R A H V P P P E V T E G F V L A W R R A A R E Q L R E
 GACGCGCAGCCGCTCCCCAGCGGCAGCAGATCCGACTTCAAGAACGCCGGAGGAGAGGCCGGCACGGGGCGGAGCTGGC 960
 V R L R T G L P L L D S K L F A P P S A P V P R L Q G
 D A Q P R P Q R Q Q I R L Q E R R R R G R H G A E L A

CGCGCGTGTAGAGGGAGCGGGACTTCGCCGATGGATCCGGCCATGCGGATCCGCTCCTCGATGCCAGGCTCGCCGCCAG 1040
R T Y L S R S K A S P D P W A S G S R S A W A R R W
A R V E G A G L R R W I R P C G S A P R C P G S P P

CTCGCGGTGGCCGGTTCTCTGTCAAGGAAGCTGTTGGGCTCTGTCTCCGTAGAGAACCCATCACGTTGTCTGGTCTGGA 1120
S A R P R N E T L S A T P D Q R R L S G M
A R A W P V L C Q G S C W V L S P .

TCACCCGCCAGGTGGGCACCGGCTTCCAGCACGACCGCATCCTTTCCACGTGAGCGGAGGCGATGCCGCCAGCGCGAGC 1200
M R Q R E

CCGGTGATGCCTCCTCCGACGACGGCAATGACGGCCATAACCGTACTCCTGGGTCTGCGTGCCTTCCTCCTTCGAGGC 1280
P G D A S S D D G N D G H N R Y S W V C V P S L L R G
TGGCGCGGGTCAAGCATCACATGGGTTGTGCTACAGCGTGCAGCGCGGTTAATACCCCATGCGCTACGCCATCTC 1360
W R A G Q A S H G L C L Q R A T R V N T P M R Y A I S
CGGTGCCAGCGTGGCATCGGTCTCGAATTCGTCCGGCAACTTCTCGAACGTGGCGACACTGTGGAGGCGGGGGTCCGAG 1440
G A S R G I G L E F V R Q L L E R G D T V E A G V R
TTCCACGGAGGCCCGGTTGCTCTCCCGCTGATGAACAGCGTCGGCCCTCGCCTGCGGATTCACGAGCTCGACATCACC 1520
V P T E A R L L S P L M N S V G P R L R I H E L D I T
AACCAGGCCAGTGTGCGTGCCTTCGCTCGGCCGTGAGCGACGGTCCGCTGGATGTGCTCATCAACAACGCAGGGGTGAG 1600
N Q A S V R A F A S A V S D G P L D V L I N N A G V S
CGGCAAGTGGTGTCTCTTCATGGAGATGGACTACGAGGACATGACGAAGGTCATGGAGACCAACTCCGTGGGCCCCATGC 1680
G K W C S F M E M D Y E D M T K V M E T N S V G P M
GTCTGTCCGCCGCGCTGATGCCCGGGTGTCAAGGGCCCCACGCGGAAGATCATCCACCTGACCACGCGCATGGCCTCG 1760
R L S A A L M P A V L K G P T R K I I H L T T R M A S
CTCACGGAGAACACCCGGGGCGGCTCTATGGGTTGAGGGCGGGCATATGCCTACCGCATGTGGAAGGCCGCGTTGAA 1840
L T E N T R G G V Y G F E G G A Y A Y R M S K A A L N
CGTGTGCATGCGCACCATGGCCGTGGACTTCCGGGACAGGGGCTCATCACCGCGGCGATCAACCCGGGGTGGGTGCGCA 1920
V C M R T M A V D F R D Q G L I T A A I N P G W V R
CGGAGATGGGCGCAAGCTGGCGCCGATGCGTCCAGAGGACCGCTCCGGGGATGTTGCGCGTCATCGATGACATCACC 2000
T E M G G K L A P M R P E D A V R G M L R V I D D I T
W

AAGGAGCAGAGCGGCATGTTCCCTCGACTTCCAGGGCCGCGAGGTGCCCTGGTAGGGCAGGGGGCATGGGCGGCGCTCAGG 2080

K E Q S G M F L D F Q G R E V P W .
P A S R C T G R S G P G R P A R T P C P A H A A S L

GGTAGAGCAGCTCGGTGTGCCAGGCGCCGCCCTCGCGAAGGTAGACGAGGCGCTCGTGCAGGCGGCTGGGGCGGGCG 2160

P L A A R H A L R R R G R S P L R P A R A P P Q P P R

TGCCAGAACTCGATGCGGTCCGGCACCCACGCGCAGGCCGGCCAGTGCGGCGGGCGGGGCACGGGGCGCCCTCGTGCTG 2240

A L V R H P G A G R A P R G L A A P P A R P R G R A T

TTTCGTACGGCGTCCACCCGCGCTCCAGGAGTTCGGGGAGGGCAGCGGCTGGCTCTGGAGGCTGGCCACGCGCCCA 2320

E D R R G G A G G P T G P L A A A P E P P Q G V R G

GCTGGCTGCCCCGGGCGGGCTGTGGAAGTAGGCGTTCGATTCTTCCTCGGAGACGCGCTCCACGCGCCCTCGATGCGG 2400

A P Q G P R P Q P L L R E I R G R L R A G R A G R H P

ATCTGCCGCTCCAGGGGGGCCAGTAGAAGACGAGCGCGGCGAAGGGCTGGCCGAGCAAGCTCCTCGGCCCTTGCGGCT 2480

D A A G P P G L L L R A R R L A P R A L E E A G Q P Q

GGTGAGGTTGGTGAAGAAAGACGAAGCCTCGCGCATCGAAGTCCTTGAGGAGGACGACGCGCGGGAAGGGCGGCCCTCG 2560

H P Q H L F V F G R A D F D K L L V V R A S P R G E

GCCCCACCGAGGCGACGATCATGGCGTTCGGATCCACGGGAATGGCCTTTTTTCGCTGCTCGTACAGGGTGGCGAAGCG 2640

A G V S A V I M A N P D V P I A K K A Q E Y L T A F R

TTCGATGGGGTCTGGGGCAGTTCATGACGCGCACCTAACAAATCCCGCTCGCCGAGTGAAGGCCGGGGTTGCGCACGT 2720

E I P D Q P L E M

TCTATGGTTGACTCATGGGCGCTACACGGCATGTTTCGGGCATGAAAATCCTGTTTCATCGCCTCGGAAGTCACCCGTTT 2800

TCCAAAACGGGCGGACTGGGCGACGTGGCGGGGGCACTGCCCGCGGCGTTGCCGCACTGGGTGATGACGTCAAGGTGGT 2880

CAGCCCCCGCTATGCGGAGGTGAAGGATGCCCGGCTCACGCCACGGGTAAGTCTGCTGCTGCGCTTTCCCTTTGGCA 2960

CCCCGGGGGGCCCCATTCTGTCCGTGCGGCTCTCGGAGCGGCACGAGCTGCTCTTCTCGAGCAAAC 3027