Monitoring and adapting to changes in environmental conditions are critical processes that determine the survival of microorganisms in natural settings and their successful long-term competition for a given habitat. Our group focuses on the physiological and molecular mechanisms by which microorganisms respond to changes in the environmental osmolarity and growth temperature. We use the Gram-positive soil bacterium *Bacillus subtilis* and taxonomically closely related genera as our model systems.

A special focus of our work is the uptake and synthesis of compatible solutes and their physiological role as stress protectants in the adaptation of *Bacilli* to increases in the external osmolarity and changes in growth temperature. This group of organic compounds – also referred to in the literature as “chemical chaperons” – is used by a wide variety of *Bacteria* and *Archaea* to offset the detrimental effects of high salinity and osmolarity on cell physiology and growth. Compatible solutes contribute to cellular survival under high salinity growth conditions by two different mechanisms: (i) Microorganisms amass these organic osmolytes either via synthesis or uptake in response to increases in the external osmolarity. They can accumulate them in molar concentrations without detrimental effects on cell physiology. The accumulation of compatible solutes by the microbial cells leads to the inflow of water and as a result, dehydration of the cytoplasm is prevented and turgor pressure is maintained under osmotically unfavourable conditions. (ii) Compatible solutes also help to preserve the native conformation of proteins and thus prevent their denaturation under conditions of high ionic strength.

In addition to their well-studied function in cellular osmoprotection, it has recently become clear that compatible solutes also help microbial cells to wither suboptimal growth temperatures. Our work with *B. subtilis* has shown that the uptake of compatible solutes is a very effective cellular defence against the negative consequences on cell physiology when *B. subtilis* is cultivated near its lower (15°C) or higher (52°C) border of growth temperature.

The task of cellular adjustment of *B. subtilis* to hyperosmotic growth conditions is accomplished in two phases. Initially, large quantities of potassium (K⁺) are accumulated through effective transport systems. We succeeded in the past to identify two K⁺ uptake systems (KtrAB and KtrCD) in *B. subtilis* that are primarily responsible for this task. In the second phase of the osmotic adjustment, K⁺ is partially expelled from the cell via unknown exporters and replaced by compatible solutes. Both synthesis and uptake from environmental sources contributes to the accumulation of compatible solutes under high osmolarity growth conditions. Increased synthesis of the amino acids glutamate and proline and the production of the trimethylammonium compound glycine betaine from the external precursor choline are used by the *B. subtilis* cell to raise the internal concentration of compatible solutes. In addition, our genetic and physiological studies have identified six transport systems operating in *B. subtilis*, that together allow the uptake of 13 different naturally occurring compatible solutes when the cells are osmotically challenged by high salinity. Accumulation of compatible solutes, either via synthesis or uptake, allows the adjustment of *B. subtilis* to unfavorable osmotic conditions and growth at high osmolarity.
The identification of *B. subtilis* as a proline producer under conditions of osmotic stress prompted the question whether most other members of the genus *Bacillus* also synthesise proline as their dominant endogenous osmoprotectant. We therefore conducted a detailed study of compatible solute synthesis at high salinity in a large number of *Bacilli* by natural abundance $^{13}$C-NMR spectroscopy. Among the 26 microbial species that we have investigated for de novo synthesis of compatible solute, we detected five classes: (i) those that synthesise only glutamate; (ii) those that produce glutamate and proline; (iii) those that make glutamate and ectoine; (iv) those that synthesise glutamate, ectoine and hydroxyectoine and finally (v) those that produce glutamate, ectoine and proline.

More than half of the *Bacilli* that we investigated for compatible solute production are capable to synthesise the heteropyrimidine ectoine as a major compatible solute. Consequently, our analysis showed that the ability to synthesise ectoine is a widespread trait among the *Bacilli*. We studied ectoine production in detail at the physiological and molecular level in *Sporosarcina pasteurii*, *Salibacillus salexigens* and *Virgibacillus pantothenticus* and found that ectoine synthesis is under osmotic control in each of these organisms and is dependent on an evolutionarily highly conserved *ectABC* gene cluster.

A subset of the ectoine producers that we have investigated, also synthesise a hydroxylation product of ectoine, 5-hydroxyectoine. We found that hydroxyectoine formation in these organisms is primarily a stationary phase phenomenon, suggesting the synthesis of hydroxyectoine probably does not only serve an osmotic function for the cell. Indeed, in the halophilic bacterium *Chromohalobacter salexigenes*, hydroxyectoine biosynthesis has been connected with the development of a resistance against high temperature stress (J. Bacteriol. 188:3774-3784; 2006).

Different groups have already studied the biosynthesis of ectoine at the molecular and biochemical level in a variety of microorganisms so that the ectoine biosynthetic route and the three enzymes involved in its formation are now well understood. However, the enzymatic conversion of ectoine into hydroxyectoine was largely unexplored. We therefore set out to identify the structural gene (*ectD*) for the ectoine hydroxylase (EctD) and to biochemically characterise the encoded enzyme. For these studies we used the moderate halophile *Salibacillus salexigens* as model organism and isolated from osmotically challenged cells the ectoine hydroxylase via several chromatographic steps to homogeneity. We found that the ectoine hydroxylase is a member of the non-heme-containing iron(II)- and 2-oxoglutarate-dependent dioxygenases. We also succeeded in isolating the *ectD* structural gene from *S. salexigens* using a molecular approach. Database searches with sequenced bacterial genomes revealed that the *ectD* gene is present in many microbial species and that these microorganisms always also contain the *ectABC* gene cluster. Often, the *ectD* gene appears to be co-transcribed with the *ectABC* ectoine biosynthetic genes but this is not the case in *S. salexigens*.

EctD-type proteins are evolutionarily closely related and form a novel subfamily within the superfamily of the non-heme-containing iron (II)- and 2-oxoglutarate-dependent dioxygenases (EC 1.14.11). In collaboration with Dr. K. Reuter (University of Marburg), we recently succeeded in crystallizing and solving the X-ray structure of the *S. salexigens* ectoine hydroxylase with a bound iron atom. Attempts are now underway to obtain an EctD crystal structure containing the substrates ectoine and 2-oxoglutarate to reveal the atomic details of the active site of the EctD enzyme.

In addition to making major contributions to the maintenance of turgor, compatible solutes also function as protein stabilisers both in vitro and in vivo. This aspect of the physiological function of compatible solutes is generally explained in terms of the “preferential exclusion model”. This hypothesis predicts that compatible solutes are excluded from the immediate hydration shell of proteins resulting in a preferential hydration of protein surfaces. The resulting non-homogeneous distribution of compatible solutes within the cell water provides a thermodynamic force driving the protein to occupy a smaller volume. This reduces the amount of excluded water and thereby stabilises the native structure of proteins and favours the formation of protein assemblies.

According to the “preferential exclusion model”, compatible solutes such as glycine betaine do not make direct contacts with the protein surface. Yet in components of transport systems - in particular the extracytoplasmatic ligand binding proteins from microbial ABC transport systems - very specific and high-affinity interactions between a protein and compatible solutes take place to permit import into the cell. To elucidate the mode of the specific binding of compatible solutes to substrate binding proteins from ABC-transport systems, we collaborated with the laboratories of Prof. L. Schmitt (University of Düsseldorf) and Prof. W. Welte (Univers-
University of Konstanz) to crystallise the OpuAC ligand binding protein from the OpuA transporter from B. subtilis. Together, we succeeded in the elucidation of a high-resolution X-ray structure of the OpuAC protein with either glycine betaine or proline betaine bound as the substrate.

Our physiological studies and transport assays have shown that both glycine betaine and proline betaine are efficient osmoprotectants for B. subtilis and are substrates for the OpuA transporter. The conducted structural work permitted a detailed view into the ligand binding pocket of the OpuAC protein and revealed that cation-π interactions between the OpuAC protein and the ligands are key for substrate binding (Fig. 1).

![Fig. 1. Crystal structure of the glycine betaine binding protein OpuAC from Bacillus subtilis. Molecular details of the co-ordination of glycine betaine within the substrate binding pocket of the B. subtilis OpuAC protein.](image)

Such cation-π interactions are also central determinants for the high-affinity binding of glycine betaine and proline betaine by the Escherichia coli and Archaeoglobus fulgidus ProX proteins. The crystal structure of both ProX proteins were previously solved in a collaboration between our group and that of Prof. W. Welte (University of Konstanz). In the meantime, we have carried out a detail mutagenesis study of the substrate-binding pocket of the OpuAC protein to assess the contribution of individual amino acids to ligand binding. Those amino acids that our combined crystallography and mutagenesis study revealed as being critically involved in ligand binding, are completely conserved among a large family of OpuAC-type binding proteins from a diverse set of microbial species. Hence the structural and molecular analysis conducted by us and our collaborators on the structure and function of the B. subtilis OpuAC protein can now serve as a model system for many microbial glycine betaine/proline betaine binding proteins.

The structural work carried out so far by us and our collaborators on ligand binding proteins from ABC-type transporters for compatible solutes has focussed on glycine betaine/proline betaine binding proteins. To further broaden our insight into the molecular determinants that govern specific compatible solute binding by a ligand binding protein of an ABC-type transporter, we again collaborated with the group of Prof. L. Schmitt (University of Düsseldorf) to obtain a high-resolution structure of a ectoine/hydroxyectoine binding protein (EhuB). This protein was derived from an ABC-transporter from Sinorhizobium meliloti that primarily functions for the uptake of ectoine and hydroxyectoine as a nutrient. Its ligands ectoine and hydroxyectoine are chemically not related to glycine betaine or proline betaine.

The overall crystal structure of the EhuB protein is very similar to that of many other periplasmic ligand-binding proteins: it features two separate lobes that are connected by a flexible hinge region. The substrates ectoine or hydroxyectoine are bound in a deep cleft that is formed by the two lobes of EhuB. In this respect the overall EhuB structure is very similar to the B. subtilis OpuAC protein. Ligand binding is again accomplished primarily via cation-π interactions between defined aromatic side chains of EhuB and either ectoine or hydroxyectoine. The substrate is further stabilised within the binding pocket by salt bridges and hydrogen bonds (Fig. 2). Site-directed mutagenesis was used by us to assess the contributions of individual amino acids within the binding pocket to substrate binding. Again, cation-π interactions between aromatic side chains and the ligands were key for high-affinity substrate binding by the EhuB protein. Interestingly, in the course of this work, we were able to create EhuB mutants that bind ectoine and hydroxyectoine with a substantially higher affinity then the wild-type protein, thereby generating super-binding EhuB variants. The combined data from the structural and mutagenesis work allowed us to make predictions about the substrate specificity and affinity of EhuB-related proteins from various Gram-negative bacteria.
We also conducted in collaboration with the group of Prof. L. Schmitt (University of Düsseldorf) a structural analysis of a choline and acetylcholine binding protein (ChoX). This protein was derived from an ABC-transporter from *Sinorhizobium meliloti* that primarily functions for the uptake of choline and acetylcholine as nutrients. Like in the *B. subtilis* OpuAC protein and the ProX proteins from *E. coli* and *A. fulgidus*, a box of aromatic amino acids is key in substrate binding of the trimethylammonium head-group of choline and acetylcholine via cation-π interactions. Interestingly, such an aromatic ligand binding box was recently also reported by G. Gadda and co-workers (Biochemistry 47:243-246; 2008) for the choline oxidase from *Arthrobacter globiformis*, an enzyme involved in the conversion of choline into the compatible solutes glycine betaine.

Under high osmolarity growth conditions (minimal medium with 1 M NaCl), *B. subtilis* can accumulate via the high-affinity OpuA, OpuC and OpuD transporters glycine betaine to a intracellular level of 1.3-1.4 M. This high-level accumulation of glycine betaine allows *B. subtilis* to grow quite effectively in high-salinity media. However, the very same compound that aid *B. subtilis* under high osmotic conditions, becomes a threat to cellular survival when the cells are rapidly subjected to an osmotic down-shock. Such an osmotic down-shock occurs in natural settings when the soil bacterium *B. subtilis* is suddenly confronted with rainfall or washout into fresh-water environments. Under these conditions, water is rapidly entering the cell along the osmotic gradient, thereby driving up turgor to unphysiological high values. This threatens to disrupt the protective cell envelope and thereby kill the cell. *B. subtilis* thus needs to engage in active countermeasures to reduce the osmotic potential of its cytoplasm.

It is known from the analysis of several bacterial species, that mechano-sensitive channels are used as safety valves to unspecifically release cytoplasmic solutes upon an osmotic down-shock. We identified in *B. subtilis* the structural genes for both a MscL- (mechanosensitive channel of large conductance) and a MscS-type (mechanosensitive channel of small conductance) channel. Disruption of these genes leads to *B. subtilis* mutants that no longer can effectively manage the rapid transition from high- to low osmolarity environments. The MscL type channel makes the greatest contribution to cellular survival under osmotic down-shock conditions. The MscS-type channel seems to be used by the cell primarily under special circumstances since the transcription of its structural gene is under the control of the master regulator (SigB) of the large general regulon of *B. subtilis*.

In addition to the already well-studied function of compatible solutes as osmoprotectants in *B. subtilis*, we discovered through physiological experiments that these organic osmolytes also serve both as heat-stress (52° C) and chill-stress (15° C) protectants. The function of compatible solutes as temperature stress protectants becomes apparent, when *B. subtilis* is continuously cultured at either its upper or lower boundary of growth. Under such adverse condition, the cells acquire from environmental sources a wide spectrum of compatible solutes via transport systems and this permits effective adaptation and cellular growth under both low and at high temperature.

We have shown, that the same transporters that take up compatible solutes for osmoprotective purposes, also allow their uptake under heat-stress and chill-stress. However, there is an important difference in amount of compatible solutes accumulated by the cells under osmotic or temperature stress growth conditions. Both at high (52° C) and at low (15° C) growth temperatures, only moderate amounts of compatible solutes are amassed by the cell, whereas – as outlined above – exceedingly high concentration of these organic osmolytes are accumulated under osmoprotective conditions. We speculate, that the low concentrations of compatible solutes (also known as „chemical chaperons“) present in either heat- or chill-stressed cells are sufficient for the stabilisation of thermolabile proteins thus aiding growth of the *B. subtilis* cell at its upper and lower temperature limits. In contrast, high concentrations of compatible solutes are
required to counteract the massive loss of cell water at high salinity. Taken together, our findings demonstrate that the role of compatible solutes extends well beyond their traditionally recognised roles as osmostress protectants for \textit{B. subtilis}. They also highlight the physiological function of compatible solute transport systems in the development of stress resistance against drastic osmotic and temperature challenges.

The lower growth limit of the wild-type and sequenced \textit{B. subtilis} strain 168 in a chemically defined medium is 13° C. We have taken a „systems biology“ approach to comprehensively define the cellular response of \textit{B. subtilis} cells continuously cultured under chill stress (15° C) conditions. For this purpose, we collaborated with the group of Prof. U. Völker (University of Greifswald) and analysed both the proteome and transcriptome of chill-stressed \textit{B. subtilis} cells. A massive re-programming of both transcription and translation takes place in chill-stressed \textit{B. subtilis} cultures and major effects on the biosynthetic capacity of the cell are noticeable. We are now studying the genetic and physiological function of selected members of chill-induced genes of \textit{B. subtilis} to reveal their contribution to cellular survival and growth under unfavourable conditions. Particular emphasis in this analysis is placed on those genes whose transcription in induced by both by osmotic- and chill-stress.

\section*{Publications}

\subsection*{Original papers}


\subsection*{Reviews}

\subsection*{Theses}

\subsection*{Dissertations}


Hadzhieva, Teodora (2007) Transcriptional activation and sensing properties of DegS-DegU: a two-component system involved in the osmotic regulation of \textit{Bacillus subtilis}. 

Diploma theses (Diplomarbeiten)


Bachelor theses


Invited lectures

University of Munich (2006)
BASF Chemical Company (2006)
University of Göttingen (2006)
University of Karlsruhe (2007)
Humbold-Universität Berlin (2007)

Structure of the group (12/2007)

Group leader: Prof. Dr. Erhard Bremer

Secretary: Heike Homberger

Postdoctoral fellows: Dr. Ina Budde, Dr. Jan Bursy, Dr. Tamara Hoffmann

PhD/Diploma students: Helena Barzantny, Tobias Bönig, Marina Höing, Daniela Hahn, Marco Pittelkow, Tim-Martin Seibert, Christian Ziert

Bachelor students: Dominik Bloes, Jessica Jüngel, Benjamin Über

Technical assistants: Jutta Gade, Maritha Lippmann, Alexandra Oulds*, Jochen Sohn. Daniela Störmer*

*part of the reported time

Grants

SFB 395 “Interaktion, Anpassung und katalytische Fähigkeiten von Bodenmikroorganismen”; fundet by the DFG: Support for 3 graduated students.

International Max-Planck Research School: Support for 1 PhD student.

Fonds der Chemischen Industrie

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