Summary

The main topic of this thesis is the regulation of transcription of the \textit{citM} gene of \textit{Bacillus subtilis}, encoding the major metal citrate transporter. CitM belongs to a small family of secondary transport proteins, the MeCit family, that is comprised of 12 members. CitM mediates the transport of metal citrate (Me$^{2+}$:citrate$^{3-}$) across the cell membrane in an electrogenic pmf-driven manner, meaning that at least two protons are cotransported. The divalent metal ions that can be taken up in complex with citrate include magnesium, manganese, nickel, cobalt, and zinc.

Expression of \textit{citM} is subject to several forms of regulation, summarized in Figure 1. First of all, expression is induced by the substrates to be translocated, which are citrate and isocitrate (Chapter 2 and 4). Induction is dependent on the action of the two-component system, CitS-CitT, which senses the presence of the inducers in the growth medium. It turned out that during growth on minimal medium \textit{citM} expression was highest when citrate was the only carbon and energy source present. The carbohydrates glucose, glycerol, and inositol were preferred over citrate, resulting in higher growth rates and a strongly repressed expression of \textit{citM} (Chapter 2). The expression of \textit{citM} was also repressed during growth on the non-sugars succinate and glutamate, albeit to a lesser extent.

A closer look at the promoter region of \textit{citM} revealed that it contains a catabolite responsive element, \textit{cre} sequence, with very little deviation from the consensus sequence, suggesting that \textit{citM} is subjected to carbon catabolite repression (CCR) mediated through the CCR components CcpA, HPr and Crh. Mutant strains deficient in these components revealed that repression could indeed be (partially) relieved. Besides glucose repression also the repression by inositol and succinate/glutamate appeared to be CCR-dependent, indicating that the CCR system reacts to signals derived from PTS sugars (i.e. glucose), non-PTS sugars (i.e. glycerol), and also non-sugars (i.e. succinate/glutamate). Furthermore, when glutamate was added in addition to glucose, a synergistic effect leading to increased repression was observed. During growth on inositol, HPr and Crh were exchangeable, meaning that either one of them was able to confer CCR-mediated repression as was illustrated by the unchanged \textit{citM} expression in the respective single mutant strains. In contrast, during growth on succinate/glutamate derepression was observed only in the \textit{crh} deficient mutant (Chapter 6), indicating that HPr could not substitute for Crh. It suggests that the CCR system can discriminate between the two proteins depending on the growth
conditions. In addition to its regulatory function in CCR, HPr is part of the phosphoenolpyruvate:sugar phosphotransferase system (PTS), which catalyzes the uptake of sugars with concomitant phosphorylation. During this phosphorylation cascade HPr becomes phosphorylated at a histidine residue (His15), whereas it is phosphorylated at a serine residue (Ser46) for regulatory purposes. The state of phosphorylation of HPr which depends on the presence of PTS sugars links its role in sugar uptake to its regulatory function. Thusfar, the HPr-like protein, Crh, has only been shown to play a role in gene regulation and a specific function for Crh is still elusive. The reason for the presence of a second HPr-like protein in Bacilli is not yet clear (Chapter 6). One can speculate that activation of HPr and Crh for gene regulation (i.e. phosphorylation at the serine residue) depends on different metabolic signals. CCR-mediated repression by succinate/glutamate is remarkable and the catabolites or signals that activate the pathway under the latter conditions are unknown. Possibly, Crh plays a more specific role in the repression during growth on these growth substrates. In Chapter 6, the distribution of Gram-positive type of CCR and, more particularly, of Crh-mediated signal transduction is described.

In this thesis, the expression of the *citM* gene was studied by using a downstream fusion of the reporter gene *lacZ* coding for β-galactosidase to the promoter region of the structural *citM* gene (PcitM-*lacZ* fusion). In Chapter 3 a model was presented that gives a quantitative account of the relation between rate of expression and level of expression. Analysis of the PcitM-*lacZ* promoter fusion by measuring the specific β-galactosidase activity of the cells demonstrated that in growing cells (exponential growth phase) the cell-specific β-galactosidase increased gradually and reached a maximum when cells entered the stationary growth phase. The level of expression was well described by the model, indicating that the increase merely reflects the pre-steady-state of expression, which is solely dependent on the growth rate. The level of expression is determined by both the rate of transcription and the growth rate.

Growth studies on rich medium (LB) revealed opposing results in that the addition of citrate did not give rise to *citM* expression during the exponential growth phase (Chapter 5). Furthermore, only a low level of *citM* expression was observed during the transition phase, which subsequently increased further in the stationary phase. The lack of expression implied the interference of repressing agents. Growth experiments using spent medium confirmed the presence of such compounds, since expression was elevated. Extensive investigation led to arginine being the effector. In addition, supplementing ornithine led to similar repression characteristics. Repression of *citM* by both arginine and ornithine was not relieved in the CcpA deficient strain, hence, CCR was not involved in arginine repression. Instead, the regulatory protein RocR, involved in regulation of the arginine catabolic genes, was
engaged in arginine repression, but only throughout the transition phase of growth. At the same time, the repression during the exponential growth phase turned out to be at least dependent on other compounds besides arginine, since derepression was noted in a ccpA mutant. Accordingly, the regulation of citM expression in rich LB medium involves two distinct mechanisms (Chapter 5).

CitM is essential for the growth on citrate, as shown by the fact that a CitM deletion strain could not grow on citrate as the sole carbon source (Chapter 4). The same applied to isocitrate, a tricarboxylic acid that was also able to induce citM expression. The addition of (extra) magnesium to the growth cultures increased the growth ability on both citrate and isocitrate, which was not caused by the metal itself. This observation evoked the suggestion of isocitrate being transported by CitM in a metal- or at least magnesium-dependent fashion. From exchange studies with E. coli membrane vesicles containing the B. subtilis CitM protein, it was apparent that isocitrate is actively transported by CitM and that the actual substrate is likely to be d-isocitrate. Uptake studies with radioactively labeled nickel (63Ni) performed in whole cells of E. coli containing CitM revealed that probably the complex of d-isocitrate with divalent metal ions is the true substrate.

The data described in this thesis demonstrate that the expression of citM is strictly controlled by the medium composition. The reason for this strict control may be that the presence of CitM in the cell membrane forms a potential hazard to bacterial cells living in environments that contain toxic metal ions.

Figure 1. Summary of regulation mechanisms affecting citM expression.