Analysis of alkali-inducible genes of *Bacillus subtilis*

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To My Wife

With respect and love

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1 Zusammenfassung

Mit Hilfe der DNA-Macroarray-Analyse konnte gezeigt werden, dass mehr als 80 Gene nach einem Alkali-Schock induziert werden (Wiegert *et al.,* 2001). Während die meisten dieser Gene unter der Kontrolle des alternativen Sigma-Faktors σ^W stehen, wird die Expression der anderen Gene von einem oder mehreren unbekannten Regulator(en) kontrolliert.

Im Rahmen dieser Arbeit wurden die Alkali induzierbaren Gene *kipR, yvdT* und *pspA* analysiert, die aufgrund ihrer Signatur für Transkriptionsregulatoren codieren und daher in der Regulation anderer Gene beteiligt sein können. Während die Gene *kipR* und *yvdT* für einen Transkriptionsregulator der IcIR- und TetR/AcrR-Familie codieren, besitzt das *pspA*-Gen Ähnlichkeit zu einem Gen eines Anti-Aktivators aus *E. coli*.

In der Northern-Blot-Analyse konnte gezeigt werden, dass alle drei Gene nach Alkalischock induziert werden. An Hand von Primer-Extension-Experimenten wurden die jeweiligen Transkriptionsstartpunkte identifiziert. Upstream von den drei potentiellen Transkriptions-Startpunkten befinden sich DNA-Sequenzen mit Ähnlichkeit zu σ^A-abgängigen Promotoren.

Mit einem DNA-Macroarray-Experiment wurden solche Gene identifiziert, die unter der negativen Kontrolle des potentiellen Anti-Aktivators PspA stehen. Nach artifiziell verstärkter Produktion von PspA wurde die Expression von mehreren Genen mindestens dreifach reduziert. In einem Far-Western-Blot Experiment wurde ein Protein mit einer molaren Masse von etwa 50 kDa identifiziert, welches mit PspA wechselwirkt.

In weiteren Experimenten wurde versucht die Frage zu beantworten, warum das *pst*-Operon (*pst* steht für **p**hosphate-**s**pecific **t**ransport) als einziges Mitglied des Pho-Regulons durch Alkali induziert wird. Die Gene dieses Operons codieren für ein Phosphat-Aufnahmesystem unter Phosphat-Mangelbedingungen. Durch Northern-Blot-Experimente konnte gezeigt werden, dass alle Gene dieses Operons Alkali-induzierbar sind. Eine Analyse des potentiellen Transkriptionsstartpunkts mittels eines Primer-Extension-Experiments ergab, dass dieser identisch ist mit dem Startpunkt der unter Bedingungen von Phosphathunger bestimmt worden war. Die Transkription des *pst*-Operons ist σ^A-abhängig und steht sowohl bei Phosphathunger als auch bei Alkalischock unter der positiven Kontrolle des PhoP-PhoR Zweikomponentensystems.

Phosphataufnahme-Experimente haben gezeigt, dass der Transport von radioaktivem Phosphat unter Alkalischock-Bedingungen drastisch reduziert ist. Dies führt offensichtlich selektiv zur Induktion des *pst*-Operons, wobei die Beobachtung, dass der aktivierte Response-Regulator eine besonders hohe Affinität für die Bindungsstelle upstream des *pst*-Promotors hat, hier vermutlich eine besondere Rolle spielt.

1 Summary

Using the DNA macroarray technique, it could be shown that more than 80 genes induced after alkali shock (Wiegert *et al*., 2001). While most of them are under the control of the alternative sigma factor σ^W, the remaining genes are under the control of one or more unknown regulator(s). By their signature, two of them *kipR* and *yvdT* code for regulatory proteins, while *pspA*, member of the σ^W regulon, encodes another potential regulator.

In this doctoral work, the genes *kipR, yvd and pspA* were analyzed. The *kipR* and *yvdT* genes code for a transcriptional regulator of the IcIR and TetR/AcrR family while the *pspA* encode a transcriptional anti-activator in *E. coli* **.**

In Northern blot analyses, it could be shown that all three genes are induced after alkali shock. The transcription start points of the *kipR* and *yvdT* genes were identified by primer extension experiments, and it appeared that the transcription is dependent on a vegetative sigma A-like promoter.
To identify genes which are under the negative control of the transcriptional anti-activator PspA, a DNA macroarray experiment was carried out. It turned out that several genes are repressed by a factor of at least three under conditions of PspA overproduction.
By using the Far-western blot technique, a protein which might interact with the PspA protein was identified. This protein has a molecular weight approximately 50 kDa.
In addition, expression of the *pst* operon (*pst* stay for phosphate-specific transport) was analyzed which is induced after phosphate starvation and after alkaline shock. The genes of this operon are involved in the phosphate transport into the cytoplasma.
By Northern-blot experiments, it could be shown that all genes of this operon are alkali-inducible. When the transcriptional start point was determined by primer extension, it turned out to be identical to the one determined under phosphate limitation. This transcription start point is preceded by a typical σ^A-type promoter. Furthermore, alkali-induction is dependent on the PhoP-PhoR two-component signal transduction system. Phosphate-uptake experiments revealed that the uptake of inorganic phosphate was completely abolished after increasing the external pH value.

2 Introduction

2.1 The bacterial stress response

All living cells display a rapid molecular response when they are subjected to adverse environmental conditions such as nutritional depletion, oxygen availability, oxidative agents and sudden changes in temperature, pH and osmolarity. This so-called stress response results in a transient induction of a subset of genes that encode proteins which display an important protective and homeostatic function to cope with the physiological and environmental stress at the cellular level (Morimoto *et al*., 1990). Different mechanisms for the regulation of stress genes at the transcriptional level have been described and they all follow a common scheme (Fig. 1). In the absence of stress, cells are in the prestimulus or default state. Challenge to environmental stress is perceived by a specific sensor which directly or indirectly influences the activity of a transcriptional regulator. This results in either activation of a transcriptional regulator (a transcriptional activator or alternative sigma factor), or, alternatively, by inactivation of a transcriptional regulator, a transcriptional repressor, which in turn leads to the induction of a subset of genes encoding stress proteins. This induction is transient and certain mechanisms are able to modulate the expression rate of stress genes almost down to the default state present before the shock.

Some of the underlying mechanisms are highly specific and respond to one specific stressful situation, therefore designated as ‘specific stress response’. Others are more general, are triggered by different stress signals and protect the cell to a variety of stressful situations, therefore called ‘general stress response’(Hecker and Völker, 1998). In the following, I will shortly review first the general stress response of *E. coli* and *B. subtilis* and then turn to the heat and pH stress responses as examples of specific stress responses.

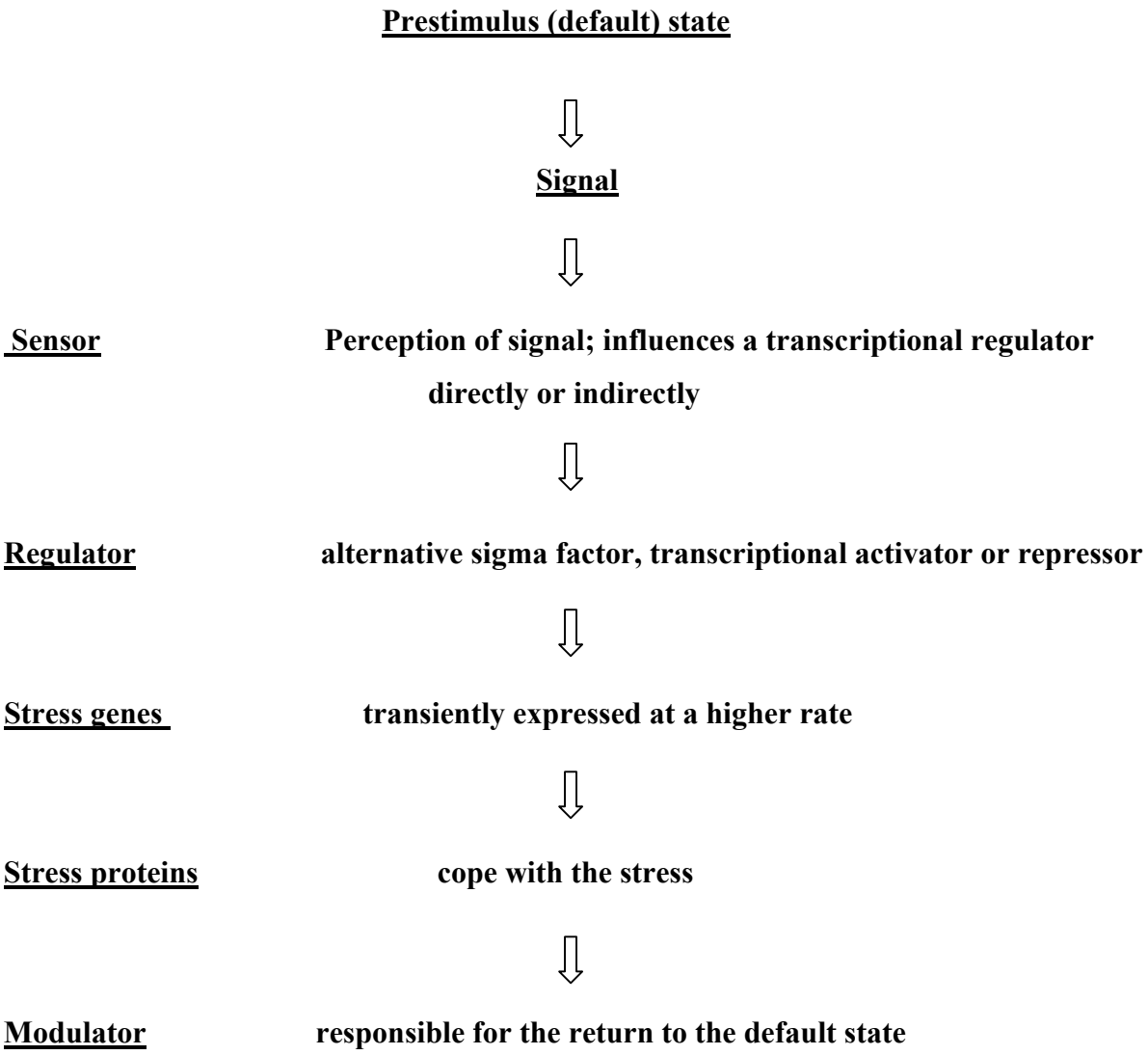


Fig. 1. Schematic representation of the stress-induced transcriptional activation

2.2 The general stress response in bacteria

The general stress response in *Escherichia coli* provides an intriguing model system to study transcriptional and translational control of gene expression, regulated proteolysis, and signal transduction integrated in a complex genetic network, which results in the drastic change in cell physiology that occurs in response to many different stresses (Hengge-Aronis *et al*., 2000). The general stress response of *E. coli* is characterised by numerous alterations in

cellular physiology and even morphology (Hengge-Aronis *et al*., 2000), and is usually accompanied by reduced growth rate or entry into stationary phase. However, σ^S rapidly accumulates in response to many different stress conditions. These include starvation for sources of carbon, nitrogen, or phosphorus or for amino acids (Gentry *et al*., 1993); shift to high osmolarity or under high osmolarity conditions (Hengge-Aronis *et al*., 1991, 1993; Kassen *et al*., 1992; Yim *et al*., 1994); shift to acid pH (Bearson *et al*., 1996; Foster *et al*., 1995).

The general stress response of *E. coli* is dependent on an alternative sigma subunit of RNA polymerase, σ^S (encoded by *rpoS*). σ^S is similar to the vegetative σ^{70} (encoded by *rpoD*) in terms of structure and molecular function (Hengge *et al*., 2002). Some σ^S-dependent genes exhibit similar heat shock induction, whereas others are not induced probably because they need additional regulatory factors that might not be present under conditions of heat shock or exponential growth (Muffler *et al*., 1997).

The σ^S subunit is subject to multiple regulation at the transcriptional, translational and post-translational level. At the post-transcriptional level, the activity of σ^S is modulated by the ATP-dependent protease ClpXP (Schweder *et al*., 1996), which consists of a regulatory component, ClpX, and a proteolytic component, ClpP (Gottesman *et al*., 1993, Wojtkowiak *et al*., 1993). The degradation of σ^S requires an additional protein, RssB (**r**egulator of **s**igma **S**) that is homologous to the group of response regulator proteins (Bearson *et al*., 1996; Muffler *et al*., 1996; Pratt and Silhavy, 1996; Andersson *et al*., 1999) and acting as a targeting protein. Genetic evidence showed that RssB is required for σ^S degradation but not for another ClpXP substrate, the O protein of phage λ, which indicates that RssB specifically targets σ^S for degradation (Zhou and Gottesman *et al*., 1998).

For the regulation of *rpoS* transcription previously data have been shown that the level of σ^S is very low during the exponential growth phase (Arnqvist *et al*., 1994). Studies with the transcriptional *rpoS::lacZ* fusion indicated that the *rpoS* transcript accumulates during the late phase and entry into the stationary phase when cells are grown in rich medium (Lange *et al*., 1995; 1994; Takayanagi *et al*., 1994). This result suggested that most environmental signals that induce σ^S do not affect the level of the *rpoS* transcript (Gentry *et al*., 1993; Lange *et al*., 1995). In addition, regulation of *rpoS* translation has been revealed using a *rpoS::lacZ* gene

fusion (Lange *et al*., 1994; Loewen *et al*., 1993; McCann *et al*., 1993). It was demonstrated that the translation of the *rpoS* mRNA is stimulated by a shift to high osmolarity or low temperature as well as during the late exponential growth phase (Lange *et al*., 1994; Muffler *et al*., 1996; Sledjeski *et al*., 1996). After the onset of starvation, *rpoS* translation is reduced again and the continuing increase in σ^S level is then due to enhanced stability of σ^S in the cells (Lange *et al*., 1994).

In *B. subtilis*, stress proteins are induced in response to different environmental conditions as heat shock, salt stress, glucose, starvation, and oxygen limitation or oxidative stress (Völker *et al*., 1994). The induction of these stress proteins is an important component of the adaptaional network of a non-growing cell of *B. subtilis* (Hecker *et al.,* 1996). The stress protein have been grouped into general stress proteins (Gsps) and heat-specific stress protein (Hsps). In addition, extracellular signals induce a set of specific stress proteins where many confer specific protection against a particular stress factor (Hecker *et al*., 1996).

In *B. subtilis*, the general stress response is controlled by the σ^B transcription factor (Price, 2000). This alternative sigma factor is activated during the stationary growth phase by a regulatory network responsive to stationary-phase signals (Boylan *et al.,* 1993b). σ^B was found to increase 5- to 10-fold when cultures were shifted from 37 to 48°C (Haldenwang *et al*., 1993). This transcription factor is required for the induction of more than 100 genes after the imposition of a whole range of stresses and energy limitation (Völker *et al*., 1999).

The σ^B is a stable protein (Redfield *et al*., 1996) whose activity is negatively controlled by the association of a specific anti-sigma factor, RsbW (for **r**egulator of **s**igma **B**) (Alper *et al*., 1994; Benson *et al*., 1993a). RsbW is a negative regulator of σ^B activity, and RsbV is a positive regulator and RsbV requires RsbW in order to exert its positive function (Benson *et al*., 1992; 1993b; Boylan *et al*., 1992). RsbW is also a protein kinase which can phosphorylate RsbV. When cells are stressed, RsbW binds to unphosphorylated RsbV, produced from the phosphorylated form of RsbV by two phosphatases RsbU, which is required for the transmission of environmental stress signals or RsbP, which is required for the transmission of energy stress signals (Delumeau *et al*., 2002; Vijay *et al*., 2000; Voelker *et al*., 1995; Yang *et al*., 1996). The central role of RsbV in the signal transduction pathway is underscored by the phenotype of a *rsbV* null mutant, which is incapable of activation σ^B in response to either energy or environmental stresses (Benson *et al*., 1993c; Boylan *et al*., 1993a,1992; Voelker *et al*., 1995). This result indicates that RsbV and RsbW modulate the σ^B activity at the

posttranslational level, and that RsbW is responsible for the rendering σ^B inactive in unstressed cells (Alper *et al.,* 1996; Benson *et al*., 1993c; Dufour *et al.*, 1994).

2.3 The specific stress response in bacteria

There are different specific stress responses in bacteria under investigation, for example the oxidative stress response, the stringent response to amino acid starvation and the heat shock response (see the book edited by Storz and Hengge-Aronis, 2000). In the following, the heat shock response which is the prime example for stress-specific gene regulation will be described in more detail. Then, our present knowledge about pH stress will be summarized.

2.3.1 The heat shock response in bacteria

One of the many survival mechanisms which is evolutionarily common among prokaryotic and eukaryotic cells is the heat shock response. The heat shock response is the cell's adaptation to an external stress, such as an increase in temperature. It is characterised by the expression of a set of proteins called heat shock proteins coping with the stress and allowing adaptation to this adverse condition. The signal which leads to the induction of the heat shock genes is caused by partially and fully denatured proteins collectively designated as non-native proteins. These non-native proteins exhibit hydrophobic amino acid residues at their surface normally buried in their interior which make them stick together leading to protein aggregates. If these protein aggregates reach a certain size they become life-threatening for the cell. Therefore, in order to survive cells try to prevent the formation of protein aggregates by two different mechanisms involving two different classes of proteins: molecular chaperones and ATP-dependent proteases (Morimoto *et al.,* 1990; Hecker *et al.,* 1996; Gottesman *et al*., 1997)). Molecular chaperones are proteins which are able to bind non-native proteins thereby preventing their aggregation. One subclass of molecular chaperones, the foldases, allow refolding of a non-native polypeptide chain into its native three-dimensional structure, and this process requires binding and hydrolysis of ATP by the chaperone (Hartl *et al*., 1975). The second subclass, the holdases, just bind non-native proteins but are not involved in their refolding. They may hand over these proteins to foldases. Important foldases present in all organisms are DnaK and its two co-chaperones DnaJ and GrpE and GroEL and its co-chaperone GroES. Important holdases are the so-called small heat shock proteins (Jakob

et al., 1993). ATP-dependent proteases on the other hand bind non-native proteins, unfold them completely followed by their degradation.

In *E. coli*, three different σ factors have been identified as being involved in the regulation of the heat shock response namely as σ^{32}, σ^{E} and σ^{54} (Yura *et al*., 2000). The active level of all three σ factors is transiently increased by a sudden temperature upshock, but the underlying mechanisms are quite different. In the absence of heat stress, only small amounts of active σ^{32} are present, and the amount of σ^{32} increases dramatically after a heat shock. This is accomplished by two different mechanisms: increased translation of the *rpoH* mRNA (the *rpoH* gene codes for σ^{32}) and increased stability of σ^{32}. Translation of the *rpoH* mRNA at low temperature is largely prevented by formation of a secondary structure which sequesters the Shine-Dalgarno sequence and the AUG start codon reducing binding of the 30S ribosomal subunit. This secondary structure is melted in a temperature-dependent manner (Morita *et al*., 1999a; Morita *et al*., 1999b). At low temperatures, the half-life of σ^{32} is below one min. σ^{32} is sequestered by the DnaK chaperone and presented to ATP-dependent proteases for degradation (Gragerov *et al*., 1992). After a heat shock, the DnaK chaperones are titrated by the non-native proteins allowing σ^{32} to survive. The more non-native proteins have been removed, the more DnaK chaperone molecules will become free to bind σ^{32} marking them for degradation.

The second heat shock-specific σ factor, σ^{E}, is present in significant amounts even at low temperatures. But this σ factor is kept in an inactive form by sequestration through the anti-sigma factor RseA (for **r**egulation of **s**igma **E**). This anti-sigma factor is a bitopic integral membrane protein which binds σ^{E} on the cytosolic site and a second protein, RseB, on the periplasmic site (De Las Penas *et al.,* 1997a,b). This complex is present in the absence of heat stress. If *E. coli* cells are treated with severe heat stress (around 45°C), RseB dissociates from this complex (its function is elusive) causing release of σ^{E} into the cytoplasm where it associates with the RNA polymerase core enzyme (Collinet *et al*., 2000). Next, RseA is degraded in a two-step process by two different proteases (Alba *et al.,* 2002; Kanehara *et al*., 2002).

The third σ factor affected by a heat shock (and many other stress regimen including alkali shock, see below) is σ^{54} (Model *et al*., 1997; Dworkin *et al*., 2000; Yura *et al*., 2000). This

alternative sigma factor controls expression of one operon only, the *psp* operon (Dworkin *et al.,* 1997; Weiner *et al*., 1991). This operon has been detected as being induced after infection with filamentous phages such as M13 and f1 (Brissette *et al.,* 1990) and later been shown that synthesis of protein IV is enough to induce this operon (Russel and Kazmierczak, 1993). Alternative sigma factors of the σ^{54} group need a transcriptional activator for carrying out the isomerization step from the closed to the open complex during initiation of transcription, and in the present case, the gene *pspF* located immediately upstream of the *psp* operon and transcribed in the opposite direction (Jovanovic *et al*., 1996) codes for this transcriptional activator. In the absence of heat stress, PspF is sequestered by the anti-activator PspA (Adams *et al*., 2003) which releases PspF following challenge with heat or many other stress factors including alkali stress (see below). Details of this process are still unknown.

In *B. subtilis*, heat shock genes have been grouped in six different classes so far, where each class is regulated by a different mechanism (Schumann, 2003). Class I genes (the CIRCE/HrcA regulon) encode the major chaperones DnaK-DnaJ-GrpE and GroEL-GroES, and their transcription depends on the vegetative sigma factor σ^{A} (Wetzstein *et al.*, 1992; Zuber and Schumann, 1994) and is negatively controlled by a repressor encoded by *hrcA*, the first gene of the *dnaK* operon (Yuang and Wong 1995; Schulz and Schumann, 1996). It has been suggested that the HrcA repressor is unable to bind to its operator when it is released from the ribosomes. To become active, it has to interact with the GroEL chaperonin system, and active HrcA is able to bind to its operator (Schumann, 2003; Mogk *et al*., 1997). Upon dissociation from its DNA binding sites, HrcA is again present in its inactive form and needs the GroE system to become its active form. Proteins which need to interact with GroEL have been described in *E. coli* (Houry *et al.,* 1999). Class II (the σ^{B} regulon) represents a large group of genes that are positively controlled by the already mentioned general stress sigma factor σ^{B}, and are activated by heat and other stresses, including starvation for glucose or oxygen. Class III genes (the CtsR regulon) encode some of the highly conserved Clp proteins that are negatively regulated by the CtsR repressor. Altogether, the CtsR repressor regulates the expression of six different genes organized in three transcriptional units. Three of these genes encode Clp proteins, one the negative regulator of this regulon, the CtsR repressor (for **c**lass **t**hree **s**tress **r**epressor), and the *mcsA* and *mcsB* genes (for **m**odulator of **C**t**s**R) are involved in regulating the activity of CtsR (Krüger *et al*., 2001). The fifth gene (*mcrA*) codes for a protein with a zinc-finger motif and an ATP-binding domain. The last gene (*mcsB*) encodes a protein whose about 60 amino acid residues of the C-terminus exhibit limited

similarity to ComEA of *B. subtilis* (Inamine and Dubnau, 1995). CtsR is composed of at least three different functional domains, a dimerization domain, a helix-turn-helix domain and the central glycin-rich region could be involved in heat sensing (Derré *et al*., 2000). The genes of the CtsR regulon are expressed at a low level at 37°C and strongly depressed after heat shock. This regulatory mechanism seems to be based on maintaining a certain steady-state level of CtsR at 37°C followed by a transient inactivation of the repressor upon exposure to heat stress. The steady-state level of CtsR is controlled by the ClpXP protease degrading superfluous molecules (Derré *et al*., 2000).

Class IV contains one gene only so far, *htpG*, which is induced about 10-fold both at the level of transcription and translation (Schulz *et al.,* 1997) and whose regulation involves the binding site for a transcriptional activator protein located immediately downstream of the σ^A-dependent promoter (Versteeg *et al*., 2003). Class V heat shock genes consist of two members so far, *htrA* and *htrB*, both coding for ATP-independent proteases (Darmon *et al*., 2002). Both genes are under the positive control by the CssRS two-component signal tranduction system. The CssRS two-component system is further assumed to detect secretion stress by sensing the accumulation of misfolded proteins at the membrane-cell wall interface (Hyyryläinen *et al*., 2001). Class VI comprises a group of genes whose expression is also responsive to heat stress, but the regulatory mechanism is undefined such as *ftsH* (Deuerling *et al*., 1997), *clpX* (Gerth *et al*., 1996) and *lon* (Reithdorf *et al*., 1994).

2.3.2 The pH shock response

Microorganisms commonly live in widely fluctuating pH environments. As a result, bacteria have evolved adaptive strategies designed to minimize acid- or alkaline-induced damage. Gram-negative and -positive neutralophiles utilize different as well as overlapping approaches for coping with acid stress (see review articles by Slonczewski and Foster, 1996; Hall *et al*., 1996; Foster, 1999; Schumann, 2000). Some inducible systems attempt to alkalinize the internal pH while other systems involve complex global changes in the proteome that somehow protect crucial, acid-sensitive cellular components. pH-responsive regulatory mechanisms involved in the adaptation to acid environments include alternative sigma factors whose levels change in response to acid stress and specific signal transduction systems that sense an acidifying environment, leading to dental caries by *Streptococcus mutans* in nature as one example (Kashket *et al*., 1987; Casiano-Colon *et al.,* 1988; Curran *et al.,* 1995; Marquis *et al*., 1987).

However, if they are first allowed to adapt to moderate acid or alkaline conditions before their limits are tested, one detects that cells can survive over a 1,000,000-fold range of H^+-ion concentration (Rengberg *et al*., 1993; Cirillo *et al*., 1998). This capability is important in the natural habitat(s) and pathogenic situations where pH fluctuates dramatically (Rathman, M and S. Falkow 1996). The principal defense against a one pH unit change in H^+ concentration above or below the optimum growth pH involves housekeeping pH homeostasis systems. Greater deviations away from optimum growth pH elicit the inducible acid survival systems (Foster, 2000).

2.3.2.1 The acid shock response

The ability to sense and respond to potentially lethal changes in the environmental is a trait crucial to the survival of any microorganism. The environmental threat endured by bacteria is acid stress, which is defined as the combined biological effect of H^+ ions and a weak acid concentration. Although the permeability of the membrane towards proteins is low, extreme low external pH will cause H^+ to leak across the membrane and acidify internal pH (Blankenhoren *et al*., 1999; Amaro *et al*., 1991; Foster *et al*., 1993; 1991).

The enteropathogens such a *Salmonella* and *E. coli* have the ability to adapt and survive acid stress that is fundamental to their pathogenesis. Once inside the host, these organisms encounter life-threatening levels of inorganic acid (H^+) in the stomach and a combination of inorganic and organic acid (volatile fatty acids) in the small intestine. To combat these stresses, enteric bacteria have evolved elegant, overlapping strategies that involve both constitutive and inducible defense systems (Audia *et al*., 2001). Several acid-resistance systems have been described for *E. coli,* and the three major systems are (i) acid tolerance resistance (ATR) (Chang *et al.,* 1999), (ii) acid habituation (Rowbury *et al*., 1998; 1999) and (iii) acid resistance (Small *et al.,* 1998).

In *B. subtilis*, the effect of acid stress is largely unknown. It could be already proven that the σ^B regulon is involved (Völker *et al*., 1999), but its activation following an acid shock remains elusive. One of the genes being member of the σ^B regulon is *yvrK* which encodes a oxalat-decarboxylase (Tanner *et al*., 2000; Maul *et al*., 1995). Two other genes are *gsiB* and *gspA* (Kalman *et al.,* 1990; Boylan *et al*., 1992; 1993a,b; Benson and Haldenwang *et al.,* 1993).

2.3.2.2 The alkali shock response

Genetic response systems enable bacteria to adapt to changes in its environment (Gottesman *et al*., 1984; Neidhardt *et al*., 1987; Stock *et al*., 1990). *E. coli* can grow over a wide range of external pHs (pH 5 to 9) while maintaining an internal pH within the narrow range of pH 7.4 to 7.8 (Padan *et al*., 1976; Slonczewski *et al*., 1981; Zilberstein *et al*., 1984).
The mechanisms of the pH homoestasis in *E. coli* remain unclear (Booth *et al*., 1985; Padan *et al*., 1987). It has been reported that the Na^+/H^+ antiporter system my be required for pH homeostasis in alkaline media (Ishikawa *et al.,* 1987)

In contrast to the well investigated acid stress response there is little knowledge of how bacteria respond to alkali stress. As for acid stress, the cells try to maintain a pH homeostasis, in this case by transporting protons into the cytoplasm. Intracellular pH homeostasis is above all maintained by secondary antiporters. These secondary antiporters make use of the proton-motive force generated across the cytoplasmic membrane by respiration and extrude toxic Na^+ ions out of the cell with uptake of protons (Kurlwich *et al*., 1994). For *B. subtilis*, the major cation/proton antiporter systems which catalyze electrogenic uptake of H^+ in exchange for cytoplasmic K^+ and or Na^+ are TetAL, and NhaC (Kurlwich *et al*., 1999). The NhaC antiporter system is encoded by the *mrp* (*yvfTUVDCB*) locus (Ito *et al*., 1997). However, the antiporters of *B. subtilis* and other bacteria investigated so far are expressed constitutively, and nothing is known about mechanisms that are induced after alkali stress and may help the bacteria to survive this adverse situation.

For *E. coli*, there are very few examples of alkali-inducible genes in the literature. One of them is the *pspA* gene. Previously data have been shown that the *pspA* gene which is the first gene of the *psp* operon of *E. coli* is induced after alkali shock. In *E. coli*, the *psp* operon (**p**hage **s**hock **p**rotein) is induced by several stress factors, including an alkali shock (Weiner and Model *et al.,* 1994). This operon contains five open reading frames, of which at least four (*pspA, -B, -C,* and *-E*) code for expressed proteins (Brissette *et al.,* 1990). In addition, PspA plays a negative regulatory role, and the intergral-membrane proteins PspB and PspC play a positive one (Adams *et al*., 2003). Transcription of the *psp* operon is dependent on an RNA polymerase (RNAP) holoenzyme containing the alternate sigma factor σ^{54} (Dworkin *et al.,* 1997). Like that of other σ^{54}-dependent genes, transcription of *pspA* requires activation by a protein, in this case PspF, which belong to the family of enhancer-binding proteins (Dworkin

et al., 1997, Jovanovic *et al.,* 1996). Through an ATP hydrolysis-dependent mechanism, these proteins convert the closed complex formed by σ^{54} and RNAP at the promoter into an open complex permissive for initiation of transcription (Kustu *et al*., 1991).

Another alkali-inducible gene in *E. coli* has been described. It was demonstrated that Cpx-regulated *E. coli* genes are also unregulated by elevated pH and furthermore, that *cpx* mutants displayed increased sensitivity to alkaline pH (Danese *et al*., 1998).

In addition to these genes, it has been found that the *alx* gene in *E. coli* is induced after alkaline-shift. The level of ß-galactosidase expression for *alx* increased 10-fold over the range pH 5 to pH 9 (Slonczewski *et al*., 1990). Some regulons including the porins such as OmpC show induction by external alkaline shift (Heyde *et al*., 1987). For more, the *iroA* (Foster *et al*., 1992, 1994) and *ompF* (Foster *et al*., 1994) are alkali-inducible genes.

Slonczewski and her collegues have found that lots of proteins are induced at the alkaline limit for growth (pH 9.0 to 9.2). For example, the glutamate decarboxylase, GadA, was identified as a protein which is induced at high pH during anaerobic growth (Slonczewski *et al*., 1999). Further more, the tryptophan deaminase (TnaA) is induced to a high level at pH 9 (Blankenhorn *et al*., 1999).

Most recently, it was attempted to define the *B. subtilis* alkali stress stimulon using DNA-macoarray analysis. An alkali shock to a pH value of 8.9 in the growth medium caused a transient growth arrest of the cells, during that time cells adapted to the new condition. At least 80 genes were induced under this condition by a factor of 3 or higher, the products of which are probably involved in coping with alkali stress (Wiegert *et al.,* 2001). About half of the genes induced were members of the σ^W regulon. The alternative sigma factor σ^W belongs to the family of sigma factors that control genes of extracytoplasmic function (ECF family). Most of the genes of the σ^W regulon are of unknown function, but have a probable role in detoxification and transport processes. The σ^W regulon is not only induced by alkaline stress, but also by salt shock, phage infection and certain antibiotics that inhibit cell wall biosynthesis such as vancomycin (Helmann *et al*., 2001; 2002). Most interestingly, the *B. subtilis* ortholog of the alkali induced *pspA* gene of *E. coli*, which is *ydjF*, was shown to be alkali-inducible in this bacterium, too, and was proven to be σ^W–controlled (Wiegert *et al*., 2001). It is believed that alkali-induction of the σ^W regulon is indirect and that σ^W-controlled genes are not directly involved in pH homeostasis. A σ^W knockout strain of *B. subtilis* is not

impaired in surviving an alkaline shock and behaves just like the wildtype. However, there were other genes identified by the DNA macroarray screen that were induced after alkali shock and that were not dependent on σ^W (compiled in Table 1).

Tab. 1: Induction of genes after alkali shock that are not controlled by σ^W (from Wiegert *et al*., 2001).

Gene	**Induction factor in sigW$^+$**	**Induction factor in (*sigW::neo*)**	**Function**
gltB	8.5 ± 3.4	22.5 ± 17.3	Glutamate synthase (small subunit)
hisA	8.5 ± 2.9	3.2 ± 1.4	PRF-5-aminoimidazole carboxamide ribotide isomerase
hisB	12.3 ± 6.7	4.4 ± 2.2	Imidazoleglycerol-phosphate dehydratase
hisD	7.2 ± 2.9	2.9 ± 0.9	Histidinol dehydrogenase
hisF	11.5 ± 5.0	2.5 ± 0.4	HisF cyclase-like protein
hisI	22.0 ± 14.3	4.6 ± 1.0	Phosphoribosyl-AMP cyclohydrolase
mrgA	11.1 ± 2.0	7.4 ± 1.6	mrgA metalloregulation DNA-binding stress protein
ybyB	6.4 ± 2.2	5.2 ± 0.2	Unknown
ycgM	39.8 ± 14.9	3.3 ± 1.6	Similar to proline oxidase
ycgN	13.9 ± 6.9	3.0 ± 1.6	Similar to 1-pyroline-5-carboxylate dehydrogenase
ycgO	4.9 ± 2.3	2.3 ± 0.7	Unknown
ycsG	3.8 ± 1.8	2.8 ± 0.8	(*ycsH*) similar to branched chain amino acids transporter
ycsJ	5.5 ± 0.3	3.9 ± 0.5	Similar to allophanate hydrolase
ycsK	4.7 ± 1.3	3.3 ± 0.8	Similar to hypothetical proteins
ycsO (*kipR*)	6.4 ± 0.8	4.8 ± 1.4	Similar to transcriptional regulator (IclR family)
yhaS	10.9 ± 2.0	10.7 ± 1.6	Unknown
yhaT	4.3 ± 1.3	3.6 ± 1.2	Similar to hypothetical proteins
yhaU	6.9 ± 2.1	7.3 ± 2.8	Similar to Na+/H+ antiporter
yheK	5.6 ± 1.2	24.6 ± 8.9	Similar to hypothetical proteins
ykoY	36.5 ± 22.2	6.0 ± 2.5	Similar to toxic anion resistance protein

Gene	Induction factor in sigW$^+$	Induction factor in (*sigW::neo*)	Function
yqgG	6.6 ± 1.5	4.4 ± 3.3	(*yzmB*); similar to phosphate ABC transporter (binding protein)
yqgH	2.2 ± 0.6	1.0 ± 0.5	(*yzmC*); similar to phosphate ABC transporter (permease)
yqgI	8.1 ± 1.9	3.7 ± 2.5	(*yzmD*); similar to phosphate ABC transporter (permease)
yqgJ	32.4 ± 8.9	22.3 ± 8.2	(*yzmC*); similar to phosphate ABC transporter (permease)
yqgK	9.0 ± 3.1	8.4 ± 3.0	(*yzmF*); similar to phosphate ABC transporter (ATP-binding protein)
yufN	8.6 ± 4.3	3.5 ± 1.0	Similar to ABC transporter (lipoprotein)
yufU	3.9 ± 0.6	2.6 ± 1.3	Similar to Na+/H+ antiporter
yufV	4.1 ± 0.4	2.1 ± 0.3	Similar to Na+/H+ antiporter
yvaE	6.0 ± 2.2	3.7 ± 1.0	Similar to multidrug-efflux transporter
yvdS	10.5 ± 1.8	6.4 ± 2.1	Similar to chaperonin
yvdT	12.8 ± 4.3	6.0 ± 0.7	Similar to transcriptional regulator (TetR/AcrR family)
ywqH	5.3 ± 2.3	2.3 ± 0.8	Unknown
ywqI	5.5 ± 1.4	3.1 ± 0.8	Similar to hypothetical proteins from *B. subtilis*
yxbB	18.9 ± 6.4	3.7 ± 2.4	(*yxaP*); similar to hypothetical proteins
yxbC	5.9 ± 0.7	2.6 ± 1.0	(yxaQ); unknown
yxnB	14.0 ± 5.7	2.3 ± 0.6	Unknown
yybO	3.8 ± 1.2	1.0 ± 0.2	Similar to ABC transporter (permease)
yybP	34.3 ± 11.3	13.7 ± 3.4	Unknown

This table shows all genes which are induced after alkali shock but they are not dependent on σ^W.

2.4 Goals of the present thesis

DNA macroarray analyses could show that more than 80 genes were induced at least three fold after increasing the external pH to 8.9 by the addition of NaOH. These alkali-inducible genes could be classified into two groups. While most of them are members of the σ^W regulon, the remaining genes are under the control of so far unknown regulator(s) (Wiegert *et al*., 2001).

The goals of this doctoral thesis were the followings:

1. Identification of the regulator(s) involved in the alkali-inducible genes not controlled by σ^W.

2. Identification of the partner protein of the PspA anti-activator.

3. Trying to answer the question why the genes of the *pst* operon, but not other members of the Pho regulon are induced by alkali stress.

3 Material and Methods

3.1 Bacterial strains, plasmids, oligonucleotides and antibodies

3.1.1 Bacterial strains

The bacteria strains used in the course of this work are listed in the Table 2.

Tab. 2: Summary of the bacterial strains used in this work. The name, genotypes and references are given for each strain. Strains marked with an asterisk (*) were constructed during this work.

Strain	Genotype	Reference
DH10B	F^- , *mcrA*, Δ (*mrr*, *hsdRMS*, *mcrBC*), φ80d (*lacZΔM15*, *ΔlacX74*), *deoR*, *recA1*, *araD139*, Δ(*ara, leu*)$_{7697}$, *galK*, λ^- , *rpsL*, *endA1*, *nupG*	Bethesda Research Laboratories
DH5α	*endA1, F*$^-$*,gyrA9 , hsdR17,* (rk^- , mk^-), *lacZΔM15, recA1, supE44,* λ^-*,deoR, thi-1, φ80d, Δ(lacZYA-argF)*$_{U169}$	Bethesda Research Laboratories
AA01	1012 *kipR::cat* (Cm^R)	*
AA02	1012 *yvdT::cat* (Cm^R)	*
AA03	1012 *pspA::cat* (Cm^R)	*
AA04	1012 *pstS::lacZ* (Em^R)	Prágai *et al*., 2001
AA05	1012 *pstBA::lacZ* (Em^R)	*
AA06	*trpC2 amyE*::pNK45 (*phoA-lacZ*) (Cm^R)	Prágai *et al*., 2001
AA07	168-PR (*phoR ΔBA/I::tet*) (Tc^R)	Prágai *et al*., 2001
AA08	1012 *ykoY::lacZ* (Em^R) (BSF1847)	Karin
AA09	1012 *hisI::lacZ* (Em^R)	*

AA10	101 *ycgM::lacZ* (Em^R)	*
AA11	1012*; yybP::lacZ* (Em^R)	*
AA12	1012*; yxbB::lacZ* (Em^R)	*
AA13	1012 *pit-pMUTIN* (Em^R)	Pra`gai *et., al* 2001
AA14	*pQE-30-pspA*-Ap^R	*
AA15	*pspA-pMUTIN4::*Em^R	*
amyE::neo	1012 with *neo* cassette in *amyE* (Neo^r)	Versteeg S.

3.1.2 Plasmids

The plasmid used during this work are listed in the Table 3.

Tab. 3: Summary of the plasmids used during this work. The name, phenotype and reference are given. Plasmids marked with an asterisk (*) were constructed, during this work.

Plasmids	**Phenotype**	**Reference**
pUC18	Ap^R	Vieira and Messing, 1982
pUC19	Ap^R	Vieira and Messing, 1982
pLacZ	*lacZ*, Neo^R	T. Wiegert
pMUTIN4	Erm^R	Vagner *et al*., 1998
pQE-30	His_6-tagging-vector, Ap^R	Stüber *et al*., 1990
pBgaB	pMLK83-2 with *bgaB,* Neo^R	Mogk *et al.,* 1996
pBluescript II KS	*LacZ***,** f1 *ori*, Ap^R, T7- and T3`promoter	Stratagene

3.1.3 Oligonucleotides used

Tab. 4: Summary of the deoxyoligonucleotides used in the course of this work

Num.	Name of the oligo	Use	DNA sequence
ON1	ycsJ-5`	PCR	GGCCATGAATTCTCATTCGAGTGGTGGAGGGATAT
ON2	ycsJ-3`	PCR	GGCCATAGCGCTAAACAATCGGAAGATCAGCGGAT
ON3	ycsK-5`	PCR	GGCCATAGCGCTTTCGATATACAGCTCTGGGCGAT
ON4	ycsK-3`	PCR	GGCCATAAGCTTTCTCATGGCAATGGGCGGATACA
ON5	CAT-BstEII-5´	PCR	GGCCATGGTTACCCGGATTTTTCGCTACGCTCAAAT
ON6	CAT-XmaIII 3`	PCR	GGCCATCGGCCGTTCAACTAACGGGGCAGGTTAGT
ON7	yveA-5`	PCR	GGCCAT GAATTC CTC TGT TTC AGC AAA TAT ACG CC
ON8	yveA-3`	PCR	GGCCATAGCGCTCCAGTAATGACTGACTCTCATTC
ON9	yvdS-5`	PCR	GGCCATAGCGCTGGTTCTTGTTTTATTGCAGGGC
ON10	yvdS-3`	PCR	GGCCATAAGCTTTCATTCCTAAAATGCCAGCCAGC
ON11	pspA-5`	PCR	GGCCATGAATTCGAAATCATTCCAAGCATTCCGG
ON12	pspA-3`	PCR	GGCCAT GTCGAC CGG AAG GCT TTC AAT ATT GTC CT
ON13	KipR-5`	Sequencing	GGCCATGGATCCTCCGCTGATCTTCCGATTGT
ON14	kipR-3`	Sequencing	GGCCATAAGCTTCCGGTGAACAGAGGTC
ON15	yvdT-5`	Sequencing	GGCCATGGATCCTATACCGGGCTCCCAAAAAG
ON16	yvdT-3´	Sequencing	GGCCATAAGCTTATAGAATGTTCCTTGGGCAG
ON 17	yvdT-3`	Primer extension	TCTATGGCCGCCTGCAATATT
ON18	kipR-3`	Primer extension	AGCAGCGCCATAGATTTGACT
ON19	ycsK5`	RNA-probe	ATGGCATGGTTTTTATTAGTGATT
ON20	yvdR-5´	RNA-probe	CTAATACGACTCACTATAGGGAGATGAAGATGACGT

ON21	yvdR3´	RNA-probe	GGCCATTTATGTCTGCGAATATCAATGC
ON22	pspA-3`	RNA-probe	CTAATACGACTCACTATAGGGAGATTGTCATGCA -TCTTTCTCATC
ON23	pspA-3´	RNA-probe	AAATCGCTCAAGAAGAAGCTAA
ON24	yxBB-3`	RNA-probe	CTAATACGACTCACTATAGGGAGAGGAATAACT -TTCTTCAAATGCC
ON25	yxBB-5`	RNA-probe	CTAGACATGAATTCAGGCA
ON26	ykoY	Primer extension	GGCCATGTCGTCATTTCATGGATTGTATC
ON27	ykoY-5`	RNA-probe	CTAATACGACTCACTATAGGGAGAATATACA -GCAAATAGATCGCGCC
ON28	ykoY-3`	RNA-probe	GGCCATGGATCCGTGTTCCAGTCTTGTTGACTCTC
ON29	ykox-5`	PCR	GGCCATCTGCAGGAGAAGAACCCAACCATACTCTA
ON30	ykox-3`	PCR	GGCCATGAATTCCACGTGCTTGAGGTCAAAGATCT
ON31	pstBA-5`	PCR	GGCCATGGATCCCCGGCATATTTCAATGCATGTGT
ON32	pstB-3`	PCR	GGCCATGAATTCATGCTTGATCATGTGAACGACGG
ON33	yybP-5`	PCR	GGCCATGGATCCCCAGGTCTGACTGCTCCAATTCT
ON34	yybP-3`	PCR	GGCCATGAATTCTAGCAGCGGGTAAAATTATCGGC
ON35	ycgM-5`	PCR	GGCCATGGATCCTTCGTCCTCCATGTCAATGGTGA
ON36	ycgM-3`	PCR	GGCCATGAATTCGCGGCAAGCAAAGAAGTGCTGAC
ON37	hisI-5`	PCR	GGCCATGGATCCTTCCGCGATCACCCGCTCCAGTT
ON38	hisI-3`	PCR	GGCCATGGATCCGCCGGAAAGCCCGTTTGACAC
ON39	pstA-5`	Sequencing	GGCCATAAGCTTGCAATGGCGCTCGACTTTGTTGT
ON40	pstA-3`	Sequencing	ACTTTCACCTGCATTACC
ON41	pstBA-neu	Primer extension	ACTTTCTCCTGCATTTCC

The DNA sequences recognized by restriction enzymes are underlined.

3.1.4 Antibiotic solutions

Information is given in Table 4 concerning the antibiotic solutions which were used in the course of this work.

Tab. 5: Summary of antibiotic solutions. Information is given about stock and final concentrations and the solvents used.

Antibiotic	Concentration of final solution (mg/ml)	Dissolved in	Final concentration (µg/ml)
Ampicillin	100	H_20 dest.	100
Chloramphenicol	10	ethanol	10
Erythromycin	1 or 100	ethanol	1 or 100
Neomycine	10	H_20 dest	10
Tetracycline	10	70% ethanol	10

3.2 Material and appliance

Roche: alkali-phosphatase, RNase inhibitors, T7- and T3-RNA-polymerase, DNase I

Merck: proteinase K

Sigma: RNaseA, lysozyme

BIOzym: T4 DNA ligase

3.2.1 Biochemical and Chemicals

Roche: blocking reagent, CSPD

Fulka: diethylpyrocarbonate (DEPC)

Gibco-BRL: agar, caseinhydrolysat, yeast extract

Pharmacia: ammoniumperoxodisulfate (APS)

Qiagen: Ni-NTA-agarose

Roth: ethidiumbromide, acetic acid, chloroform, acid phenol (Aqua Phenol), phenol/TE, potassium acetate, sucrose, MOPS, Tris, urea, diethylpyrocarbonate (DEPC), agarose.

3.2.2 Radioactive chemicals

Amersham Pharmacia Biotech: ^{32}P 370 MBq/ml, 10 mCi/ml

α-[^{33}P]-dATP (10 μCi/μl)

α-[^{33}P]-dCTP (10 μCi/μl)

α-[^{35}S]-dATP (10 μCi/μl)

3.2.3 Kits

BIOzym: Fast-Link-DNA-Ligation Kit

Gibco-BRL: CONCERT High-purity-Midi Kit, CONCERT Rapid-PCR-Purification-system, CONCERT Rapid-Gel-Extraction-Kit.

3.3 Methods

3.3.1 PCR

The polymerase chain reaction (PCR) can generate a large number of copies from even the smallest amounts of DNA (Saiki *et al*.,1988). This was enabled by the isolation of a thermostable DNA polymerase from *Thermus aquaticus*. During the PCR, DNA is denatured at high temperature, specific oligonucleotide primers are annealed and elongated at lower temperature in a cyclic manner.

3.3.2 Phosphate uptake assay

The phosphate uptake assay was carried out as described by Qi *et al*. (1997).

3.3.2.1 Special media

Hulett`s salts (per 1000 ml):

Trizma, 50 mM
Ammonium sulphate, 3.03 mM
Trisodium citrate, 6.8 mM
Ferric chloride, 3.04 mM
Manganese sulphate, 1.0 mM
Magnesium sulphate, 3.5 mM
Zinc chloride, 0.01 mM

Low-phosphate medium (0.42 mM) (LPM):

Hulett`s salts, 188 ml
Glucose, (50%), 4 ml
Casamino acids, (5%), 2 ml
L-Arginine, (1M), 4 ml
L-Tryptophan, (10 mg/ml), 400 µl
KH_2PO_4, (42 mM), 2 ml

3.4 Work with RNA

3.4.1 Isolation of total RNA from *B. subtilis*

The preparation of total RNA was carried out according to the ‚acid phenol' method described (Völker *et al*., 1994; Homuth *et al*., 1997).

3.4.2 RNA isolation for DNA macroarray analysis

For preparation of high quality RNA, a modified protocol, originally developed for extraction of RNA from *Saccharomyces cerevisiae* was used (Hauser *et al.*, 1998).

3.4.3 Northern-blot analysis

Northern-blot analyses were carried out to confirm the qualitative and quantitative determination of specific RNA molecules in the RNA mix. Northern-blot analysis was performed as described (Homuth *et al.,* 1999)

3.4.4 Electrophoresis of RNA in agarose gels and transfer to nylon membranes

The separation of RNA samples was run on 0.8% agarose gel and the transfer occurred on Nylon-membranes by Northern-blotting. The transfer was carried out with help of the Vacuum-Blot-Annex (VacuGene ^{TM}X1) from Pharmacia company

3.4.5 In-vitro-synthesis of DIG-labeling ssRNA-molecules (riboprobes)

Synthesis of DIG-labeled ssRNA molecules used as antisense-RNA was carried out *in vitro* using the „DIG-RNA-Labeling-Kit" (SP&/T7) from Roche company.

Tab. 6: Summary of all RNA probes that have been used in this work. The gene transcripts, the template and the RNA polymerase used during this work are given.

RNA probe	Template	RNA-Polymerase
ydjF	PCR with ydjF oligos T7 5´ and 3´	T7
yvdT	PCR with yvdT oligos T7 5` and 3`	T7
kipR	PCR with kipR oligos T7 5` and 3`	T7
pstBA	PCR with pstBA oligos T7 5`and 3`	T7
ykoY	PCR with ykoY oligos T7 5` and 3`	T7
ycsK	PCR with ycsK oligos T7 5` and 3`	T7
yvdR	PCR with yvdR oligos T7 5` and3`	T7

3.4.6 Purification of the DIG-labeling antisense-RNA probe by pre-absorption

When the DIG-labelied–antisense-RNA used at the beginning for hybridization experiments, a very strong background was detected on the X-ray-film. The more RNA probe was used, the more the background was decreased, till it totally disappeared. While this phenomenon is known, its reasons are not known (Sprenger *et al*., 1995). As a result of this, the RNA probes were purified routinely before using them in hybridization experiments. The purification was carried out as described by Homuth *et al*. (1997).

3.4.7 Hybridization of membrane-bound RNA with DIG-labeling RNA probe

The hybridization with DIG-labeled RNA probes was carried out according to the protocol as described (Homuth *et al*.,1997).

3.4.8 Stripping of RNA blots

The stripping of the RNA membrane was carried out as described (Homuth *et al.*, 1997)

3.5 Primer extension analysis

The primer extension experiment was carried out as described by Wetzstein *et al*. (1992).

3.6 Work with Protein

3. 6.1 Purification of polyclonal antibodies

If polyclonal antibodies exhibit to many unspecific cross-reactions in a Western-blot, purification of those specifically interacting with the protein of interest (in the present work the PspA protein) is possible. The principle of this methods consists of conjugating PspA to CNBr-activated sepharose and pouring the polyclonal antibodies over the column. Those which specifically interact with the PspA will be eluted.

3.6.1.1 Overexpression and purification of His6-PspA protein

10 ml of an overnight culture of strain *pspA*pQE30 (AA14) were added to 1000 ml LB medium containing 100 µg/ml ampicillin. The culture was shaken at 37°C until an OD_{600} of 0.6 was reached. A 1-ml-sample was taken immediately before induction representing the uninduced control; cells were kept on ice. Next, IPTG was added to the culture to a final concentration of 1 mM to induce for the expression of the *pspA* gene, and the culture was shaken for another 3-4 h at 37°C. The second 1-ml-sample was withdrawn (the induced control), kept on ice first, and then centrifuged. The pellet was resuspended in 50 µl 3x SDS-PAGE buffer and analyzed by SDS-PAGE for successful induction. The remaining induced cells were harvested by centrifugation at 4000x g for 20 min. The pellets were washed and resuspended in LYB solution. The cell pellet was either used directly or stored at –20 °C for further use. The pellets were sonicated 6 times for 10 seconds each with 30 seconds intervals at 200-300 W. The lysate was kept on ice during the whole experiment. The lysate was centrifuged at 10000 x g at 4°C for 20-30 min. The supernatant was decanted and kept on ice. The pellet was resuspended in 5 ml of buffer A (5 ml buffer A/g pellet). The protein extract which was resuspend in buffer A was centrifuged at 10000 rpm and the supernatant was loaded onto the affinity chromatography column. This column was previously equilibrated with 10 ml H_2O, 10 ml buffer F, 10 ml H_2O and 50 ml buffer A. The Protein (PspA) were eluted at room temperature with 80 ml buffer A, 40 ml buffer B, 40 ml buffer C, 80 ml buffer and 40 ml buffer E. After that the column was washed with 30 ml buffer A, 20 ml H_2O/0.1% NaN_3 and was stored at room temperature.

20 µl from the previous collected fractions were mixed with 10 µl 3x sample buffer. Before loading onto an SDS gel, the samples were incubated in a water bath at 95°C and finally centrifuged at 12000 rpm for 1 min at room temperature. Afterwards, the fractions which contain purified proteins were pooled together:

3.6.1.2 Coupling of the PspA protein to CNBr-sepharose beads

The pH of the collected fractions was adjusted slowly with diluted NaOH to pH 8.0. They were centrifuged for 10 min at room temperature (RT) and 5000 RPM to remove the precipitated portions. A 0.33 g CNBr sepharose was loaded onto the column for the coupling process (for each 1 mg of PspA protein, 0.11g CNBr sepharose). And then the column was equilibrated with 100 ml mM HCl, and washed with 20 ml of buffer B containing no

mercaptoethanol. About 3.3 ml (1.1 mg/ml) with the extracted protein was loaded onto the equilibrated chromatography column which contains sepharose. After that, the column was rotated for at least 4 h at RT. After this rotation step, the beads were sedimented and washed three times with buffer B without mercaptoethanol and once with 1 M ethanolamine (pH 8.0). In the next step blocking of the beads was made. To block the beads, 1 M ethanolamine solution (pH 8.0) was added to the column and all together were rotated for 1-2 h at RT. After the blocking reaction, the beads were washed as follows: 10 ml of 0.1 M Na-acetate, 10 ml 0.5 M NaCl (pH 4.0), 10 ml 0.1 M borate and 10 ml 0.5 M NaCl (pH 8.0). The column was equilibrated with 50 ml PBS (phosphate-buffer saline) mixed with 0.1 % NaN_3 and stored at 4°C.

3.6.1.3 Adsorption of the antibodies

3 ml of serum were centrifuged at 8000 x g for 20 min at 4°C and the supernatant was collected (the pellet contained the fibronectine and denatured proteins). The serum was loaded onto the column and circulated for 1 h; the elute was collected and stored at 4°C. After that, the column was washed as follows: ones with 1 ml PBS, twice with 6 ml BBS/Tween and then again with 2 ml PBS. The washed fractions were collected and stored at 4°C.

3.6.1.4 Prestripping of the column

The affinity chromatography column which contains the beads, was washed with 2 ml PBS and 1 ml glycine/HCl/dioxan (pH 2.2). The pH of the elute was tested with pH paper, and when the pH started to drop, the liquid surface on column was marked. From here the antibody elution started. The column was washed with PBS until the pH of the elute reached a value of 7.4.

3.6.1.5 Elution and dialysis of the antibodies

A mixture containing 10 ml of 2 M glycine (adjusted to pH 2.2 using diluted HCl) and 1000 µl dioxan was loaded and allowed to run through the column. 10 aliquots of 750 µl of the resulting elute are collected separately in 10 tubes contain 250 µl 1 M K_2HPO_4. To each tube, 100 µl of NGS (Normal Goat serum) are added and shacked. PBS of pH 7.4 was added to the column and the fractions were collected up to that pH and checked using pH paper. The whole

fractions were stored at 4°C. The antibody fractions were combined and dialyzed against PBS in 0.02% sodium azide solution.

Buffer A

6 M guanidine-HCl (MW 95.53), pH 8.0
0.1 M NaH_2PO_4
0.1 M Tris-HCl
10 mM β-mercaptoethanol

Buffer B

8 M urea (MW 60.04), pH 8.0
0.1 M NaH_2PO_4
10 mM β-mercaptoethanol

Buffer C

8 M urea , pH 6.3
M NaH_2PO_4

Buffer D

8 M urea , pH 5.9
0.1 M $NaH_2 PO_4$

Buffer E

8 M urea, pH 4.5
0.1 M $NaH_2 PO_4$

Buffer F

6 M guanidine-HCl
0.2 M acetic acid

PBS (phosphate-bufferd saline)

(1 liter, pH, 7.4)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

BBS-Tween, pH 8.3

0.1 boric acid
0.02 M Na-tetraborate
1.0 M NaCl
0.1% Tween 20

3.7 Overproduction and purification of His6-PspA- protein

The *pspA* was cloned downstream of an IPTG-inducible promoter and a His_6-tag coding region in the pQE30 expression vector resulting in plasmid pQE30-*pspA*. Overexpression of *pspA* was performed as followed: 10 ml of an overnight culture from the *pspA*-pQE30 strain was added to 900 ml LB medium containing 100 µg/ml ampicillin. The culture was shaken at 37ºC until an OD_{600} of 0.7 was reached. A 1 ml sample was taken before induction (non-induced sample), then the pellet was resuspend in 50 µl 3x sample buffer. IPTG was added to

a final concentration of 1 mM. The culture was shaken for 3-4 h at 37°C. A second sample was collected (induced sample), and cooled to 4°C on ice. Cells were harvested by centrifugation at 4000 x g for 20 min, resuspended in 5 ml lysis buffer The sample was either used directly or stored for purification.

3.7.1 Purification under denaturing conditions

The overexpressed PspA protein was purified by His6 tag-Ni^{2+} affinity chromatography. Cells were resuspended in 5 ml per gram wet weight of buffer A containing 6 M guanidine hydrochloride and lysed by stirring for 15 min. The lysate was cleared by centrifugation at 10,000 g for 30 min. Ni-NTA agarose was added according to the expression strength (binding capacity 5–10 mg/ml resin) and mixed by shaking for 1 h. The mixture was poured into a column, washed with buffer B and then buffer C. Proteins were eluted with buffer D and buffer E.

3.7.2 Preparation of native cell extracts from *B. subtilis*

For the preparation of native cell-extracts a suitable volume was taken from bacterial cultures and centrifuged (15 min at 3500 rpm, 4°C). The bacterial pellet was resuspended in 1/10 volume sodium phosphate-buffer (0.1 M Na-Phosphate, pH 7.0; 1 mM $MgCl_2$). The cell-disruption was performed by ultrasonication (output control 3.5-4; 50% duty cycle; 2 x 15 pulse, in between 15 sec interval). The insoluble cell constituents were resuspended (1200 rpm, 15 min, 4°C).

3.7.3 Extraction of denatured total cell extracts from *B. subtilis*

For the extraction of denatured cell extract from *B. subtilis* a certain of volume a bacterial culture was taken (according to 0.5-7 OD_{578}) and centrifuged (15 min at 3500 rpm, 4°C). The pellet was resuspended in 100 ml lysis buffer (15% (w/v) sucrose, 50 mM Tris/HCl, pH 7.2). After addition of 4 ml lysozym solution (20 mg/ml) the cells were incubated for 5 min at 37°C. Afterwards, 50 µl of 3x sample buffer were added to the suspension and frozen up for the further use. Before use the samples were heated for 5 min at 100°C.

3.7.4 Measurement of the protein concentration

The method of Bradford (1976) was used for the measurement of the protein concentration from cell extracts.

3.7.5 Protein electrophoresis using discontinuous SDS-PAGE

The electrophoretic separation of proteins according to molecular mass was performed as first described by Laemmli *et al.* (1970).

3.7.6 Measurement of the ß-galactosidase activities

ß-Galactosidase activities were measured as described elsewhere (Mogk *et al.,* 1996), with the exception that the LacZ activity was measured kinetically in a microplate reader (VersaMax, Molecular Devices) at 405 nm at 28°C. One unit was defined as E_{405} * min $^{-1}$* OD_{600}, in which OD_{600} is the optical density of the growth culture when samples were drawn.

3.7.7 Western-blot

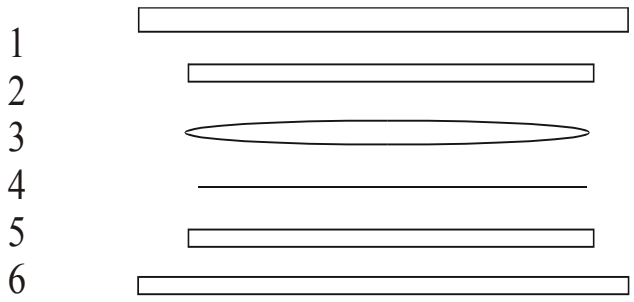
In order to immunochemically detect proteins using polyclonal antibodies, after electrophoresis, the proteins were transferred onto a nitrocellulose membrane using electroblotting (*Laemli et al.,* 1970; Towbin *et al.,* 1979; Burnette *et al.,* 1981). The electrophoretic transfer of the proteins to the nitrocellulose membrane was achieved by ,,Semi-Dry-Blotting" between graphite plate electrodes in a ,, Fast-Blot" apparatus (BioRad).

3.7.8 Far-Western blot analysis

Far-Western blot is a technique in which protein/protein interactions are studied. Proteins are run on a gel and transferred to polyvinyliden-difluorid (PVDF) membrane as in a normal Western blot (Katami, 2001; He *et al.,* 2001).

The cell extracts were denatured by boiling in sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis using 12% gels. After electrophoresis, proteins were electrotransferred onto PVDF membranes, which were then blocked with 5% skimmed milk in PBS-0.05% tween 20 (PBS-T) overnight at 4°C. Then the purified protein (PspA) produced from *E. coli* was allowed to bind to blotted proteins in PBS-T containing 5% (wt/vol) skimmed milk overnight at 4°C. The blot was washed three times with PBS-T for 30 min and was then reacted with polyclonal antibody (αPspA) in PBS-T

containing 5% skimmed milk for 1 h at room temperature. After washing, the membrane was incubated with the anti-rabbit IgG for 1 h at 4 °C (Katami *et al*., 2001). Reacted proteins on the membrane were then visualized by the enhanced-chemiluminescence system (Amerssham Pharmacia Biotech)



1: Cathode plate

2: 4-Blotting paper with cathode buffer

3: SDS gel

4: PVDF membrane

5: 2-Blotting paper with anode buffer

6: Anode plate

Fig. 2: Schematic drawing of the Far-Western-blot.

Anode buffer 1: 300 mM Tris in 20% (v/v) methanol

Anode buffer 2 :25 mM Tris in 20% (v/v) Methanol

Cathode buffer1: 40 mM ε-aminocapron acid, 0.01% (w/v) SDS in 20% (v/v) methanol

3.8 Construction of plasmids and strains

3.8.1 Construction of the three knockout strains *kipR*, *yvdT* and *pspA*

All three genes are induced by alkaline shock and code for potential transcriptional regulators. To investigate their putative influence on the regulation of alkaline-inducible genes, they were deleted from the chromosome and at the same time replaced by a *cat* cassette.

The *kipR* knockout was constructed in the following way. The flanking regions of *kipR* were amplified using ON1 and ON2 for the upstream and ON3 and ON4 (see Fig. 3A) for the downstream region resulting in two amplicons of about 300 bp which were ligated into pUC19 cut with *Eco*RI and *Hin*dIII, and both amplicons were joined by a common *Eco*47III site. Next, the *cat* cassette (generated with ON5 and ON6 and plasmid pSKII as template) was cloned into the unique *Eco*47III site resulting in the plasmid pUCΔ*kipR*. This plasmid was used to amplify a 1.6-kb fragment using the primers ON1 and ON4, and the new amplicon was transformed into *B. subtilis* strain 1012, and chloramphenicol-resistant colonies were selected.

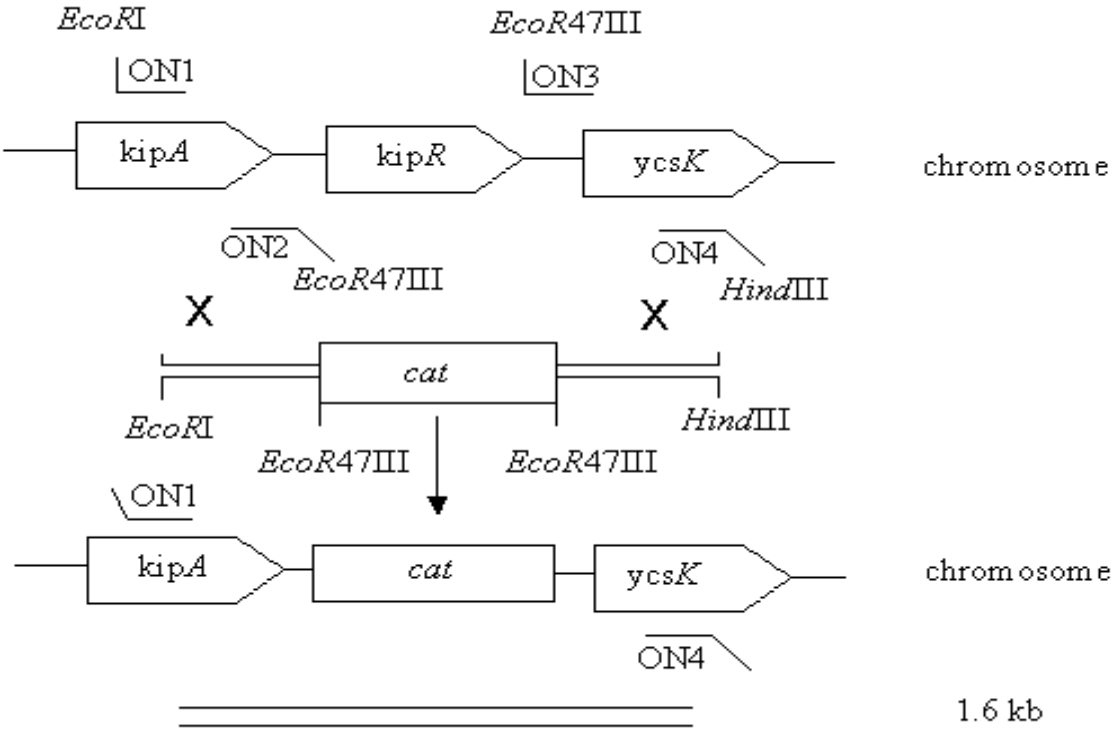


Fig. 3A: Schematic representation of construction of the *kipR* knockout.

To verify the replacement of *kipR* by the *cat* cassette, chromosomal DNA was prepared from one clone and checked with the primers ON1 and ON4. A 1.6-kb fragment was recovered (Fig. 3B).

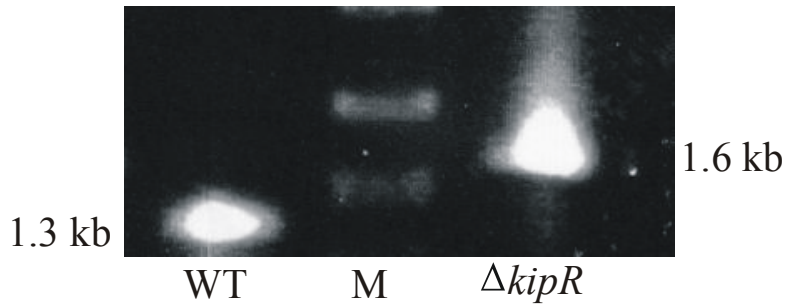


Fig. 3B: PCR analyses to prove the *kipR* mutant. PCR was carried out the chromosomal DNA from the *kipR::cat* strain. Chromosomal DNA from *B. subtilis* 1012 (WT) was used as a control. The ON1 and ON4 were used as primer. The *kipR* knockout exhibited one fragment at 1.6 kb, while the WT exhibited a fragment at 1.3 kb. M, molecular weight marker fragments.

In a second experiment, correct integration was further confirmed by Southern blotting. Chromosomal DNA from two clones and from wild-type DNA was cut with *Eco*RI, the fragments were separated through a 0.8% agarose gel, electroplotted on a Nylon membrane and hybridized with *kipR* DNA probe. While wild-type DNA resulted in two fragments of 3.6 and 1.6 kb, DNA from the two recombinant clones of only one of 5.2 kb as predicted by the DNA sequence (Fig. 3C). One of the two recombinant clones was designated as AA01 and kept for further studies.

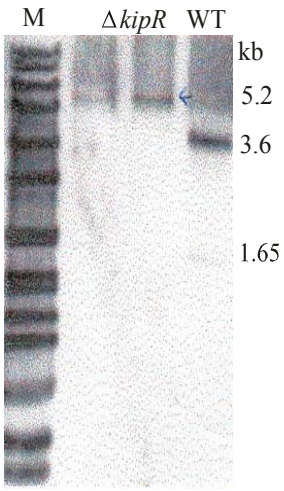


Fig. 3C: Southern-blot analyses of the *kipR* knockout. Chromosomal DNA of the *kipR* mutant and from the *B. subtilis* strain wild-type 1012 (WT) were isolated and cut with *Eco*RI. For hybridization, a *kipR* DNA probe was used. M, molecular weight marker fragments.

The *yvdT* knockout was constructed in a comparable way. First, the up- and downstream regions were amplified by PCR using ON7, ON8, ON9 and ON10, and the two amplicons were assembled into pUC19 followed by the addition of the *cat* cassette resulting in pUC19Δ*yvdT* (Fig. 4A).

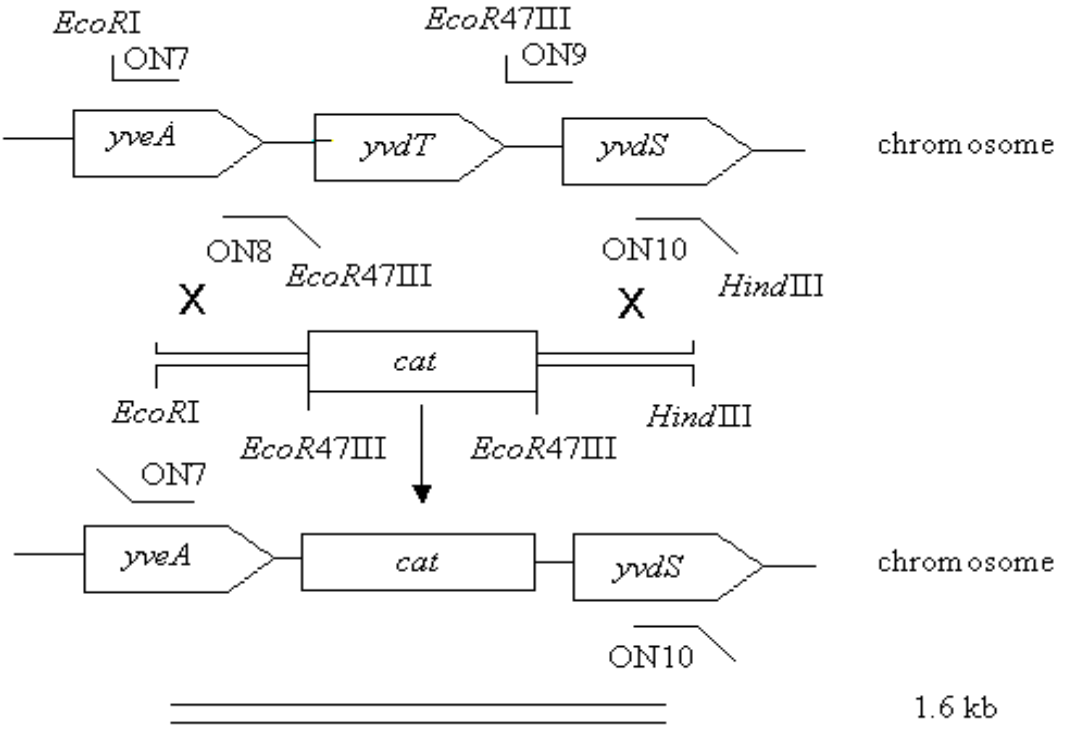


Fig. 4A: Schematic representation of the construction of *yvdT* knockout.

Using this plasmid as a template and ON7 and ON10 as primers, a 1.6-kb fragment was generated and used to transform *B. subtilis* 1012. Chloramphenicol-resistant transformants were selected and analyzed by PCR and Southern-blotting as described for the *kipR* knockout. The results of the PCR reactions are shown in Fig. 4B and reveal a 1.6-kb fragment as to be expected.

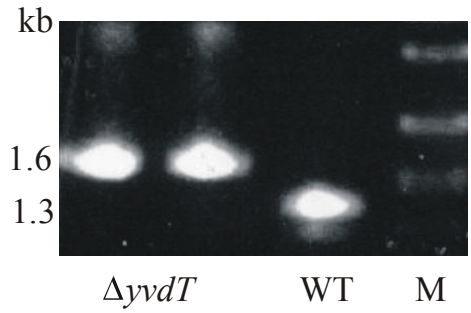


Fig. 4B: PCR analyses to prove the *yvdT* mutant. PCR was carried out using the chromosomal DNA from a *yvdT::cat* strain. Chromosomal DNA from *B. subtilis* 1012 (WT) was used as a control and ON7 and ON10 as primers. M, molecular weight marker fragments.

For Southern blotting, chromosomal DNA of the wild-type strain and of one transformant was cleaved with *Hin*dIII, and the blot hybridized with the *yvdT* DNA probe While the wild-type DNA revealed two signals with 1.43 and 1.28 kb, only one signal of about 3 kb was obtained with the recombinant DNA (Fig.4C). The strain (AA02) was kept for further studies.

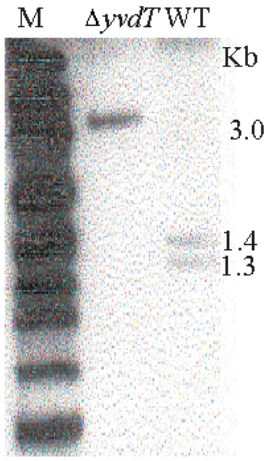


Fig. 4C: Southern-blot analyses of the *yvdT* mutant. Chromosomal DNA was prepared from *B. subtilis* 1012 (WT) and the *yvdT* knockout. Chromosomal DNA of both strains were cut with *HindIII*, and then the blot was hybridized with the *yvdT* DNA probe. M, molecular weight marker fragments.

The *pspA* knockout was constructed in a different way. Using the oligos ON11 and ON12, the complete *pspA* gene was amplified using *B. subtilis* 1012 DNA as a template (Fig. 5A). The amplicon was cleaved with *Eco*RI and *Sal*I and inserted into the pUC18 vector cut with the same enzymes.

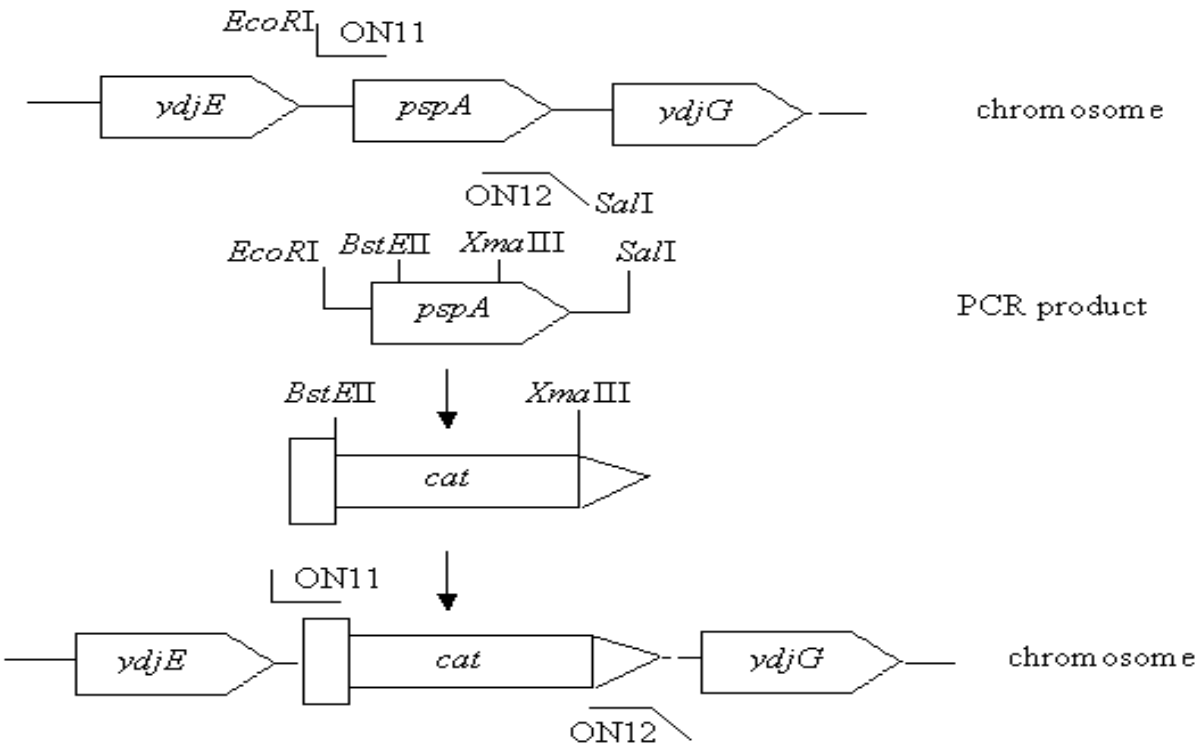


Fig. 5A: Schematic representation of construction of the *pspA* knockout.

Next, the recombinant plasmid was treated with *BstE*II and *Xma*III resulting in two fragments of 3.4 and 0.538 kb, and the smaller fragment was replaced by a 0.7.5 kb *cat* cassette generated with ON13 and ON14 and the plasmid pSKII as a template resulting in pUC18Δ*pspA*. Using the oligos ON11 and ON12 and the new plasmid as a template, a 2.7-kb fragment was obtained which was subsequently transformed into *B. subtilis* 1012. Chromosomal DNA of several transformants was prepared and tested first by PCR (Fig. 5B)

and then by Southern blotting (Fig. 5C) as described above for *kipR* and *yvdT*. Strain AA03 was kept for further studies.

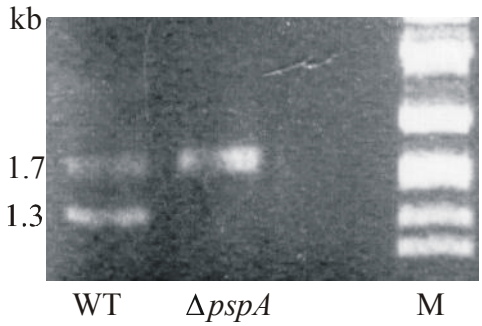


Fig. 5B: PCR analysis to prove the *pspA* mutant. PCR was carried out using the chromosomal DNA from *pspA::cat* strain. Chromosomal DNA from *B. subtilis* 1012 (WT) was used as a control. The ON11 and ON12 were used as primer. M, molecular weight marker fragments.

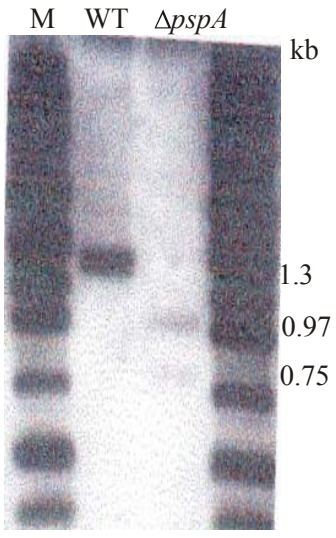


Fig. 5C: Southern-blot analyses of the *pspA* knockout. Chromosomal DNA was prepared from the *B. subtilis* 1012 (WT) and from *pspA* mutant. DNA of the both strains were cut with *XmnI*. After blotting, the membrane was hybridised using the *pspA* DNA probe. M, molecular weight marker fragments.

4 Results

4.1 Construction and analysis of knockouts in genes coding for potential regulator proteins induced by alkali shock

Three of the about 80 genes induced at least 4-fold by alkaline shock and coding for potential transcriptional regulators were chosen for further studies. These genes are designated as *kipR*, *yvdT* and *pspA*. While *kipR* and *yvdT* code for a transcriptional regulator of the IcIR and TetR/AcrR family, respectively (Wang *et al*., 1997), *pspA* has been reported to encode a transcriptional anti-activator in *E. coli* (Weiner *et al.,* 1991). Since all three genes are induced by an alkali shock, we reasoned whether at least one of them might be involved in the regulation of a subset of alkali-inducible genes, especially those not under the control by σ^W. To investigate the putative function of these three genes, knockouts were generated as described under Materials and Methods resulting in the three strains AA01 (Δ*kipR*), AA02 (Δ*yvdT*) and AA03 (Δ*pspA*). We first asked whether the knockout mutants would exhibit impaired growth after challenge to an alkali shock. Growth experiments were carried out with and without alkali shock. Cells of all three strains and of the isogenic wild-type strain 1012 as a control were grown in LB to the mid-logarithmic phase. The result of these experiments revealed that the external pH of 8.9 is not lethal to the mutant cell. The shock caused a transient growth arrest and the cells adapted to the new condition as found for the wild-type strain 1012 (Fig. 6).

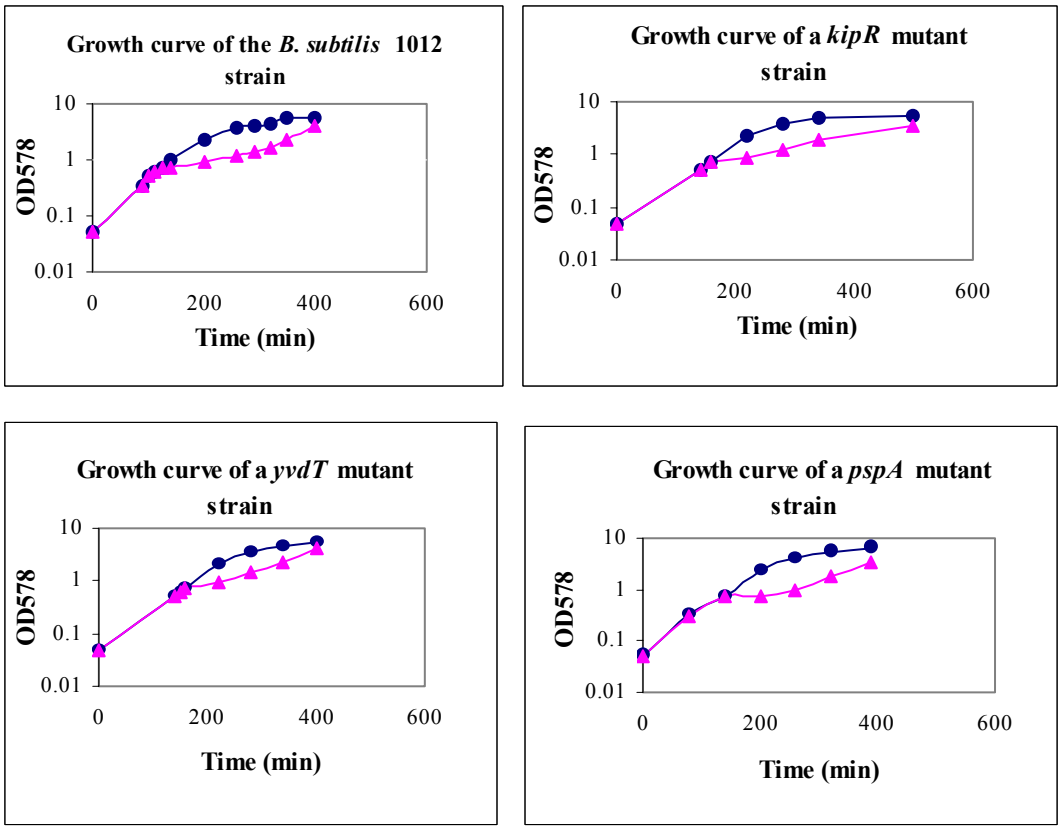


Fig. 6: Growth curve experiments. Growth curves of the *B. subtilis* strains 1012, *ΔkipR*, *ΔyvdT* and *ΔpspA*, in LB medium at 37°C without (•) and with (▲) NaOH. At an OD_{578} of 0.7, NaOH was added to the culture to the final concentration of 24 mM resulting in an external pH of 8.9.

Next, Northern-blot analyses were carried out to first confirm the alkali-inducibility of the three potential regulator genes and second to evaluate the operon structure of all three genes. From the genome sequence, *kipR* has been deduced to be part of an octacistronic operon consisting of the eight genes *ycsF – ycsG – ycsI – kipI – kipA – kipR – ycsK – yczI*. If all these genes are transcribed into one polycistronic mRNA, it should have a length of 6.0 kb. Total RNA was prepared before and at different time points after an alkali shock and hybridised with a DIG-labelled *kipR* anti-sense RNA. The result shown in Fig. 7A revealed two bands of 2.4 and 1.35 kb where only the latter exhibited a significant increase between 5 and 30 min. While the larger band could represent a transcript originating from the three genes *kipI – kipA - kipR* (the calculated size of these three genes is 2.5 kb), the smaller one could represent *kipR* and the downstream gene *ycsK* (calculated size: 1.4 kb). No signal representing all eight genes could be detected. To investigate the possibility that the 1.4-kb transcript indeed represents the *kipR – ycsK* genes, the Northern blot was repeated this time using *ycsK* antisense RNA

(Fig. 7B). It turned out that this probe revealed a signal of about 1.3 kb. Therefore, I conclude that the two genes *kipR- ycsk* constitute an alkali-inducible bicistronic operon.

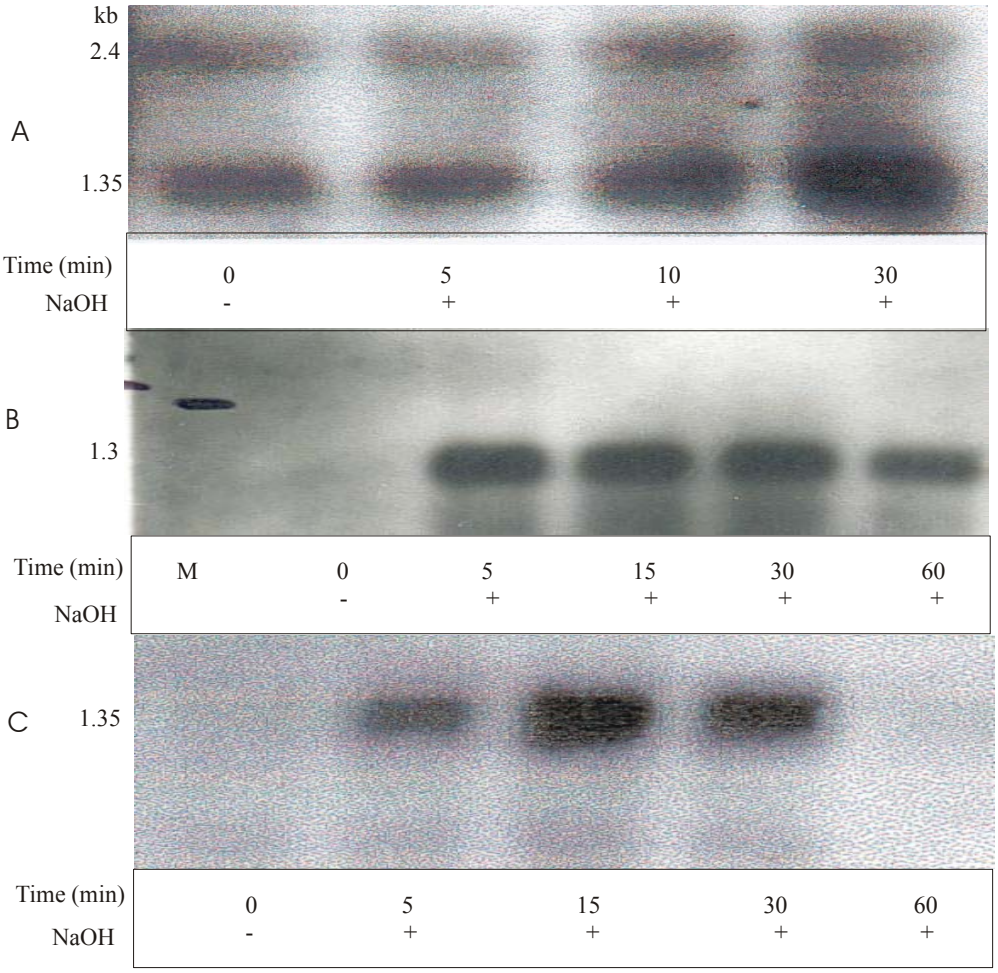


Fig. 7: Transcriptional analysis of three potential regulator genes. Total RNA was extracted from *B. subtilis* 1012 wild-type, before and 5, 10 30 and 60 min after addition of NaOH. The Northern-blots were probed with DIG-labelled antisense RNA *kipR* (A), *ycsK* (B) and *yvdT* (C). 2 µg of total RNA were applied per lane. M, molecular weight standard.

The genomic structure of the putative *yvdT* operon revealed the tetracistronic structure *yveA - yvdT – yvdS – yvdR* with calaculated size of about 3 kb. The Northern blot exhibited one band at 1.35 which strongly increased between 5 and 15 min (Fig. 7C) and could represent the three genes *yvdT – yvdS – yvdR* (calculated size: 1.23 kb). This result seems to exclude that *yvdR* is part of this alkali-inducible transcript. When a *pspA* antisense probe was used in the Northern-blot, a major signal was detected (Fig. 8). This major band with a size of about 0.7 kb most probably represents the monocistronic *pspA* transcript, consisted with the predicted structure.

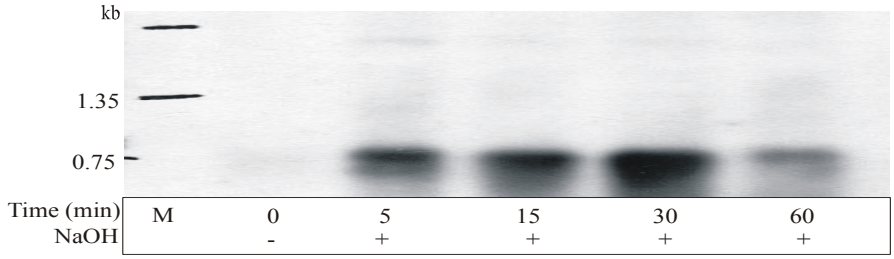


Fig. 8: Transcriptional analysis of the *pspA* gene. Northern-blot of total RNA extracted from *B. subtilis* 1012 wild-type, before (-) and 5, 15, 30 and 60 min after addition of NaOH (+). A *pspA*-specific DIG-labelled antisense RNA riboprobe was used for hybridisation. M, molecular weight standard.

Next, the potential transcription start sites for the two genes *kipR* and *yvdT* were determined by primer extension. That of *pspA* had already been published (Wiegert *et al*., 2001). Two signals of about the same strength were obtained with the *kipR* primer corresponding to an A and G in the transcript (Fig. 9), and both signals increased after alkali challenge. When the total RNA preparation was analysed with the *yvdT* primer, one major and a few minor bands became apparent, but only after alkali treatment (Fig. 10). The prominent signal corresponds to a G residue at the 5' end of the *yvdT* transcript.

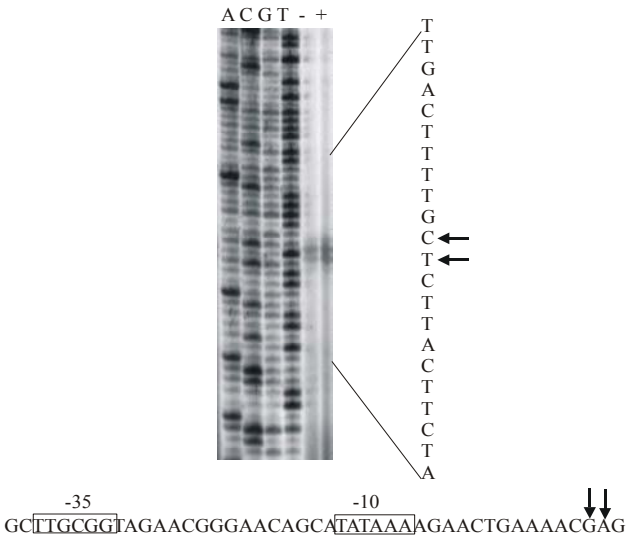


Fig. 9: Mapping of the *kipR* promoter. The 5` end of the *kipR* transcript was mapped by primer extension. Equal amounts of total RNA isolated from *B. subtilis* 1012 before and 20 min after alkali shock were used. The potential transcriptional start points are marked by an arrows. Lanes A, C, G and T show the dideoxy sequencing ladder obtained with the same oligonucleotide as used for primer extension. The location of the potential transcription start site relative to the deduced promoter is given below the experimental data.

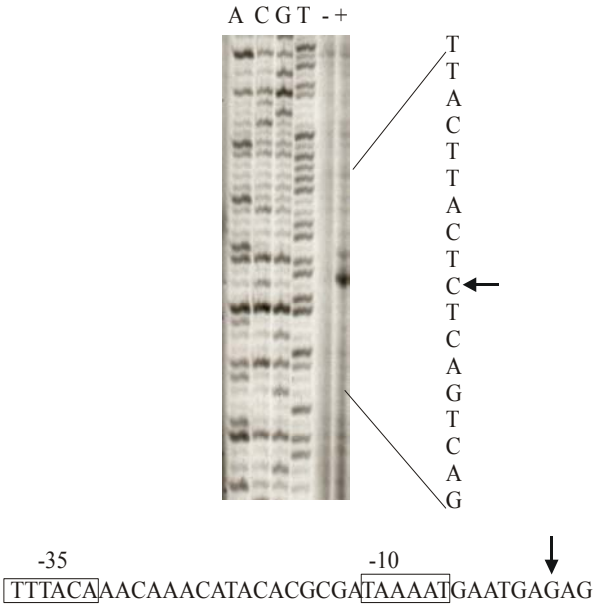


Fig. 10: Mapping of the *yvdT* promoter. The 5` end of the *yvdT* transcript was mapped by primer extension. Equal amounts of total RNA isolated from *B. subtilis* 1012 before and 20 min after alkali shock were used. The potential transcriptional start point is marked by arrows. Lanes A, C, G, and T show the dideoxy sequencing ladder obtained with the same oligonucleotide as used for primer extension. The location of the potential transcription start site relative to the deduced promoter is given below the experimental data.

4.2 Are the three potential transcriptional regulator proteins involved in the regulation of alkali-inducible genes?

As already mentioned, the three genes *kipR*, *yvdT* and *pspA* code for putative regulators, and all three genes are induced by alkaline stress. The question was asked whether one or more of these genes might be involved in the regulation of those alkali-inducible genes not being member of the σ^W regulon. To accomplish this goal, expression of the following genes was measured by Northern blotting in all three knockouts: *yhaU*, *ycgM*, *yybP, yxbB* and *hisI*. While the *hisI* and *yxbB* gense exhibited no induction in the all three knockouts strains **(**data not shown**)**, the *yhaU* gene became induced in the Δ*yvdT* and Δ*pspA* knockout strains (Fig. **11**) to the level found in the wild-type strain 1012. In addition, the *yybP* and *ycgM* seemed to enhanced induction in the Δ*pspA* strain (Fig. 12 and 13).

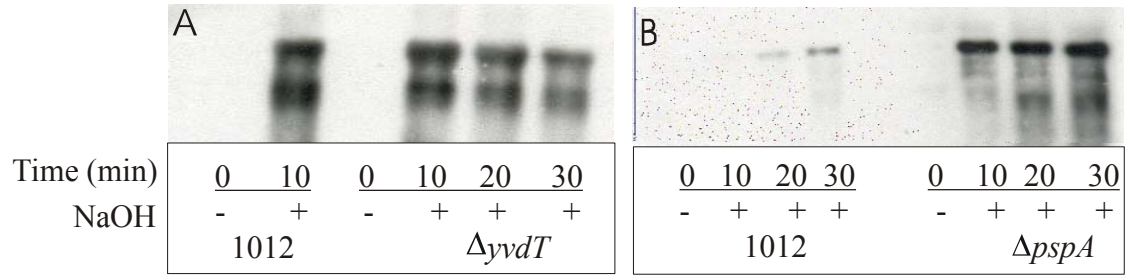


Fig. 11: Northern blot analysis of the *yhaU* gene. Total RNA was prepared from strain 1012 before (as indicated by "-" and 10 (A) or 10, 20 and 30 min after addition of NaOH (B, indicated by "+"), from strains Δ*yvdT* (A) and Δ*pspA* (B). DIG-labelled *yhaU* antisense RNA was used to detect the transcripts.

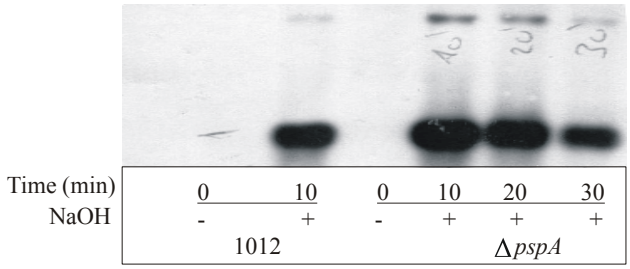


Fig. 12: Analyses of the *yybP* gene. Total RNA was prepared from strain 1012 before (as indicated by "-") and 10 or 10, 20 and 30 min after addition of NaOH (as indicated by "+"), from strain Δ*pspA*. DIG-labelled *yybP* antisense RNA was used to detect the transcript.

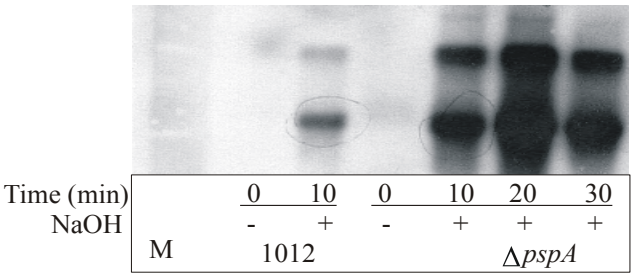


Fig. 13: Analyses of the *ycgM* gene. Total RNA was prepared from strain 1012 before (as indicated by "-") and 10 or 10, 20 and 30 min after addition of NaOH (as indicated by "+"), from strain Δ*pspA*. DIG-labelled *ycgM* antisense RNA was used to detect the transcript. M, molecular weight standard.

In summary, while alkali-induction of the *yhaU* gene was not impaired in Δ*yvdT,* it seemed to be enhanced together with the *ycgM* gene in the Δ*pspA*.

4.3 Identification of the partner protein(s) of the PspA anti-activator

In *E. coli*, the PspA protein has been reported to sequester the transcriptional activator protein PspF (Jovanovic *et al*., 1997; Elderkin *et al*., 2002) thereby acting as an anti-activator protein. Therefore, it can be assumed that PspA exerts this role also in *B. subtilis*. Two different experimental approaches were used to identify the partner protein(s), a genetic and a biochemical one. The first approach is based on the assumption that an artificial overproduction of PspA protein should result in reduced expression of those genes under positive expression of the PspA partner protein(s). It should be possible to identify these genes by the DNA macroarray technique. The biochemical approach tries to identify the partner protein(s) by the Far-Western technique.

4.3.1 The *pspA* gene, fused to an IPTG-inducible promoter can be expressed at an enhanced level after addition of IPTG

The *B. subtilis pspA* gene is induced by an alkaline shock in a σ^W-dependent manner. It displays sequence similarity to *E. coli* PspA, which negatively regulates transcription of the *psp* operon, most probably by interacting with an enhancer binding protein PspF.
I asked which genes are under the indirect negative control of PspA. Assuming that these genes are under the direct positive control of an unknown transcriptional activator, these genes should be downregulated under conditions of overproduction of PspA. To accomplish this goal, the coding region of *pspA* was fused to an IPTG-inducible promoter using pMUTIN4 resulting in strain AA15. To prove overproduction of PspA, strain AA15 was in LB medium at 37 °C. At mid-log phase, IPTG was added to the final concentration of 1 mM. Aliquots were taken immediately before and different times after addition of IPTG as indicated in Fig. 14. The proteins of these sample were separated by SDS-PAGE followed by a Western blot using PspA-specific antibodies. As shown in Fig. 14**,** no PspA could be detected in the absence of IPTG, while its amount increased after addition of the inducer. This experiment clearly shows that PspA can be overproduced.

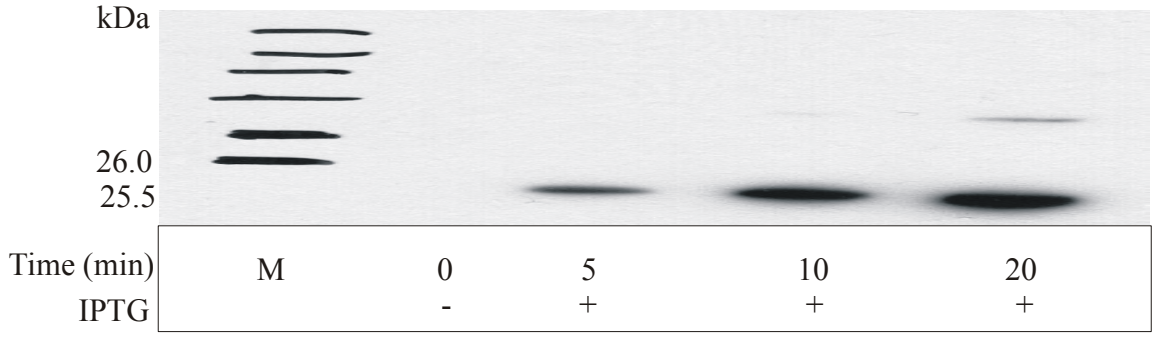


Fig. 14: Western-blot analysis of the AA15 strain using PspA antibodies. Western blot of cell extracts withdrawn from a *B. subtilis* AA15 strain at different time point before and after IPTG shock. M, molecular weight marker fragments.

To confirm this result at the level of transcription, a Northern blot was carried out using *pspA* antisense probe. Total RNA was prepared from AA15 strain grown aerobically in LB medium with erythromycin with and without the addition of IPTG. 1 mM IPTG was added to the culture at an OD_{600} of 0.7 and the sample was taken 20 min after the shock. A control sample without IPTG was drawn 20 min after reaching an OD_{600} of 0.7. The results obtained with the Northern-blot analyses prompted to ask whether additional genes are under the control of *pspA* (Fig.15). To answer this question, a survey of all *B. subtilis* transcripts was carried out using the DNA macroarray approach (Fig. 16 and 17).

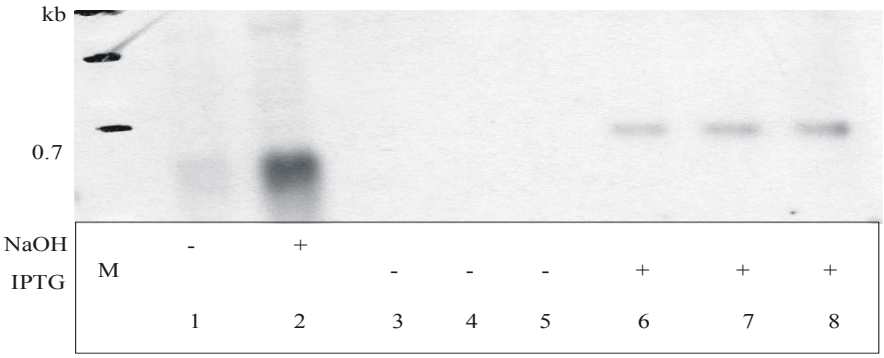


Fig. 15: Northern blot of total RNA extracted from *B. subtilis* 1012 wild-type before (-) and 20 min after alkali shock (+) (lanes 1 and 2), and from AA15 strain before (-) (lanes 3-5) and 20 min (lanes 6-8) after addition of IPTG (+). A *pspA* DIG-labelled antisense RNA was used for hybridisation. M, molecular weight marker fragments.

4.3.1.1 DNA macroarray analysis

Western-blot and Northern-blot analyses have shown that the *pspA* gene in strain AA15 was significantly induced after addition of IPTG. To identify genes being under the indirect negative control of PspA, the DNA macroarray technique was used. High quality total RNA from the strain AA15was prepared without and 20 min after addition of IPTG at an OD_{600} of 0.7. Then, the total mRNA was reverse transcribed into cDNA and labelled with ^{33}P. The cDNAs were hybridised against a complete set of PCR-amplified open reading frames. Hybridisation products were detected using a phosphorimager and quantified with the appropriate computer software (Array Vision; Imaging Research).

The result of the DNA macroarray experiments revealed that several genes were repressed at least 3-fold under conditions of PspA overexpression (Fig. 16 and 17). These genes are candidates of being under indirect negative regulation by PspA (Tab. 7). When such an analysis was carried out by optical screening, similar direct and indirect repeats could be detected with some of the genes (Fig. 18). I also tried to identify promoter sequences, but besides *cotS* which is under σ^K control, no known promoter sequence could be identified, indicating that these genes are either under the control by an unknown sigma factor or, alternatively, the genes are transcribed by σ^K but the –35 region has been replaced by a binding site for DNA-binding protein.

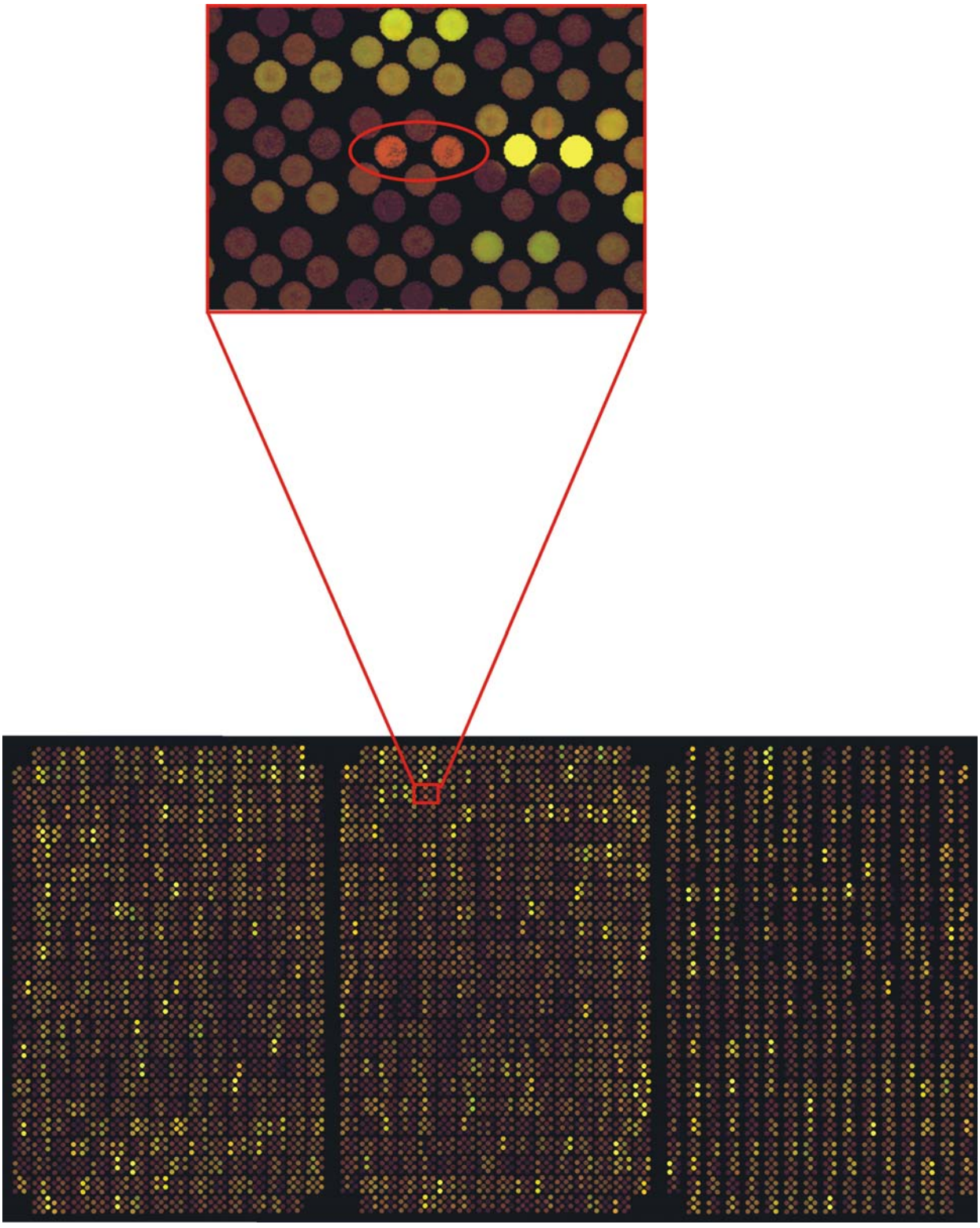


Fig. 16: The red/green images were artificially generated and serve to allow a simplified overview. Red spots represent upregulated genes, green spots downregulated genes and yellow spots represent genes with unaltered expression. The induction of *pspA* is clearly shown in the upper picture.

A

B

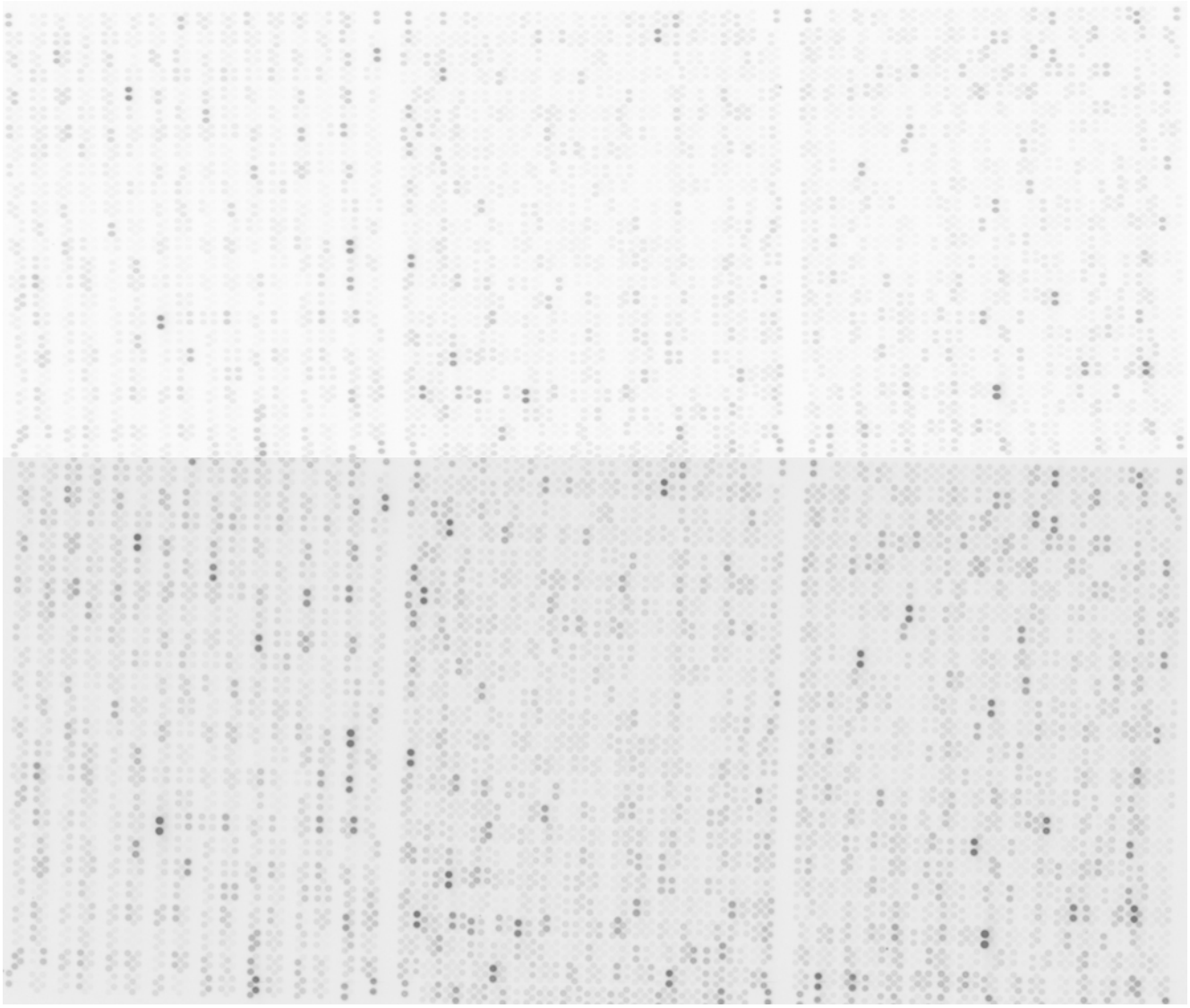
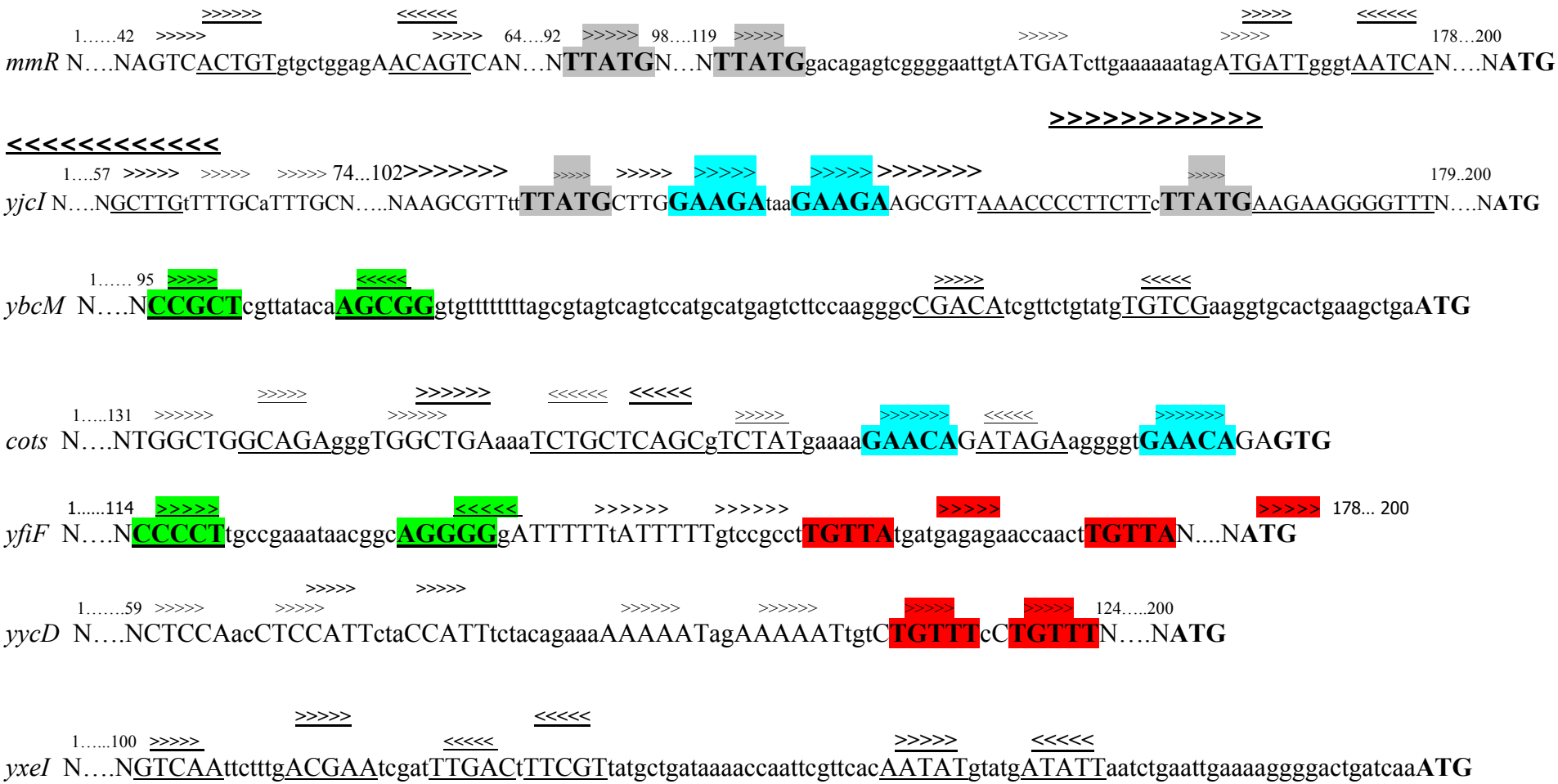


Fig. 17: DNA arrays of the entire set of *B . subtilis* genes hybridized with probes generated from RNA extracted from strain AA15 (*pspA*pMUTIN) with (A) or without IPTG (B).

Gene	Function	Comments
yycD	unknown	
yfiF	unknown	similar to transcriptional regulator (AraC/XylS family)
yorJ	unknown	
yjcI	unknown	probably part of the S box regulon; similar to cystathionine gamma-synthase
yorC	unknown	
yxeI	unknown	similar to unknown proteins from *B. subtilis*
yorM	unknown	similar to unknown proteins
ybcM	unknown	similar to glucosamine-fructose-6-phosphate aminotransferase
cotS	spore coat protein	sigma-K and GerE-dependent; located in the inner coat layer; CotE required for the assembly of CotS in the coat
mmR	Methylenomycin A resistance protein	
yhjE	unknown	similar to unknown proteins from *B. subtilis*
bglC	endo-1,4-beta-glucanase	cellulase;hydrolysing carboxymethyl cellulose
yesK	unknown	
tagC	polyglycerol phosphate assembly and export	putative; DNA-damage inducible

Tab. 7: This table shows the genes which are downregulated under PspA.


1……42 >>>>> >>>>> 64….92 >>>>> 98….119 >>>>> >>>>> >>>>> 178…200
mmR N….NAGTCACTGTgtgctggagAACAGTCAN…NTTATGN…NTTATGgacagagtcggggaattgtATGATcttgaaaaaatagATGATTgggtAATCAN….NATG
>>>>>>>>>>>>
<<<<<<<<<<<<<
1….57 >>>>> >>>>> >>>>> 74...102>>>>>>> >>>>> >>>>> >>>>> >>>>> >>>>>>> >>>>> 179..200
yjcI N….NGCTTGtTTTGCaTTTGCN…..NAAGCGTTttTTATTGCTTGGAAGAtaaGAAGAAGCGTTAAACCCCTTCTTcTTATTGAAGAAGGGGTTTN….NATG
1…… 95 >>>>> <<<<< >>>>> <<<<<
ybcM N….NCCGCTcgttatacaAGCGGgtgtttttttttagcgtagtcagtccatgcatgagtcttccaagggcCGACAtcgttctgtatgTGTCGaaggtgcactgaagctgaATG
>>>>> >>>>>> <<<<<< <<<<<
1…..131 >>>>>> >>>>>> >>>>> >>>>>>> <<<<< >>>>>>>
cots N….NTGGCTGGCAGAgggTGGCTGAaaaTCTGCTCAGCgTCTATgaaaaGAACAGATAGAaggggtGAACAGAGTG
1.....114 >>>>> <<<<< >>>>>> >>>>>> >>>>> >>>>> 178... 200
yfiF N….NCCCCTtgccgaaataacggcAGGGGgATTTTTtATTTTTgtccgccttTGTTAtgatgagagaaccaactTGTTAN....NATG
>>>>> >>>>>
1…….59 >>>>> >>>>> >>>>>> >>>>>> >>>>> >>>>> 124…..200
yycD N….NCTCCAacCTCCATTctaCCATTctacagaaaAAAAATagAAAAATtgtCTGTTTcCTGTTTN….NATG
>>>>> <<<<<
1……100 >>>>> <<<<< >>>>> <<<<<
yxeI N….NGTCAAttctttgACGAAtcgatTTGACtTTCGTtatgctgataaaaccaattcgttcacAATATgtatgATATTaatctgaattgaaaaggggactgatcaaATG

51

Fig. 18: DNA sequences located upstream of the start codon of the genes which are downregulated under conditions of overproduction of the potential anti-activator PspA. For the first six genes the similar sequences are marked by the same color. (>>->>) refers to direct repeats, while (>><<) refers to indirect repeats

4.3.2 Overproduction and purification of His-Tagged PspA Protein

The first step in the purification was the construction of a plasmid carrying a modified *pspA* gene possessing six consecutive His codons located downstream from a strong inducible promoter. To overproduce the His-tagged PspA protein, the modified *pspA* gene was inserted downstream of the IPTG-inducible promoter in the pQE30 expression vector resulting the plasmid pQE30-*pspA* strain (AA14). This plasmid was transformed into *E. cloi* XL1 blue strain which was grown in LB medium at 37 °C. Expression of the modified *pspA* gene was induced by the addition of IPTG (1 mM final concentration). Aliquots were taken before and 3 h after addition of IPTG and analysed for the synthesis of PspA using an SDS-PAGE using polyclonal antibodies. Addition of IPTG led to the increase of an about 25-kDa protein (Fig. 18, compare lanes 1 and 2) which was subsequently purified as described in Material and Methods.

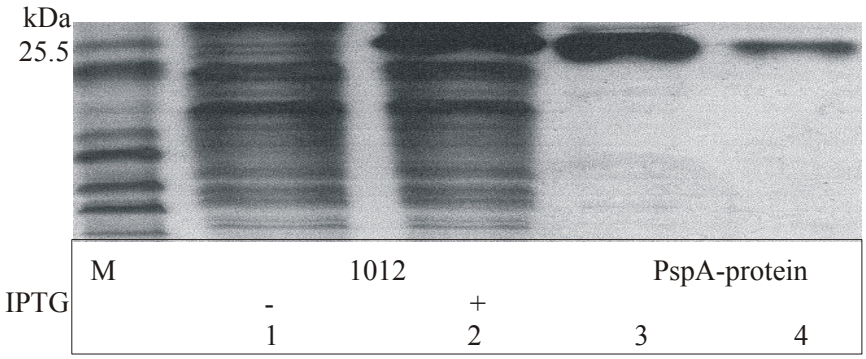


Fig. 19: Effect of induction time on the production of His-tagged PspA. Proteins of each step in the purification procedure were analyzed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane M, marker proteins; lane 1, preinduction; lane 2, postinduction at 3 h after addition of IPTG, lanes 3 and 4 fractions of the purified PspA protein with different concentrations.

4.3.3 Purification of polyclonal antibodies raised against PspA protein

His-tagged PspA was purified and used to raise polyclonal antibodies in a rabbit. It turned out that these polyclonal antibodies produced several unspecific cross-reacting bands making it difficult to identify PspA (data not shown). Therefore, the polyclonal antibodies were purified as described in Material and Methods. Next, these purified antibodies were tested in a Western-blot experiment**.** When extracts of the wild-type strain 1012 were analysed, a single band was detected that increased after addition of NaOH. In contrast, this band was absent from strain AA03 carrying a *pspA* knockout (Fig. 20). I conclude from this result that the purified antibodies detect one single protein in cell extracts of *B. subtilis* cells which due to its molecular mass and its absence from strain AA03 has been identified as PspA.

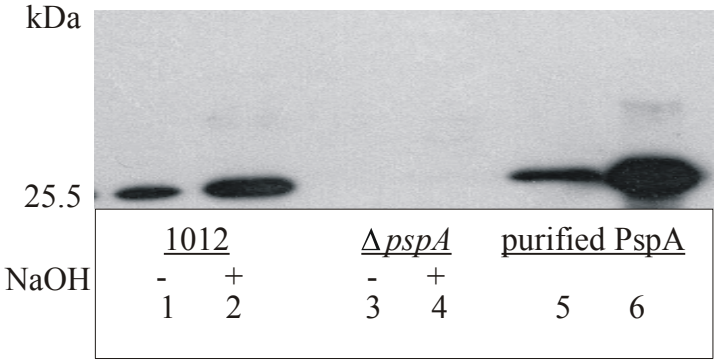


Fig. 20: Detection of PspA by purified polyclonal antibodies in a Western-blot experiment. *B. subtilis* strains 1012 (wild-type) and AA03 (*pspA* knockout) were grown in LB medium at 37 °C. Aliquots were withdrawn before and 20 min after addition of NaOH to raise the external pH to about 8.9. Lanes 1 and 2, cell extracts from strain 1012; lanes 3 and 4, cells extracts from strain AA03; lanes 5 and 6, 0.1 µg and 1 µg of purified PspA. Cells were grown in the absence (-) or after addition of NaOH (+).

4.3.4 Search for partner protein(s) by Far-Western blotting

Since in *E. coli* PspF has been identified as a partner protein of PspA (Jovanovic *et al*., 1997; Elderkin *et al.,* 2002), it is reasonable to assume that at least one partner protein exists for the *B. subtilis* homolog, too. There are several technical approaches to identify partner proteins,

and the Far-Western technique has been chosen here which has been derived from the Western-blot technique.

In the first step, proteins of cell extracts prepared from the two *B. subtilis* strains 1012 (wild-type) and AA03 (Δ *pspA*) were separated in an SDS-PAGE. In the second step, the proteins were transferred to a PVDF membrane and subsequently probed with purified PspA. In the last step, the membranes were developed with the purified polyclonal antibodies. The result of a typical Far-Western blot are shown in Fig. 21. The Far-Western technique was carried out as described in Material and Methods. The result of this experiment exhibited that additional band appears at approximately 50 kDa which is probable the partner protein of PspA. As control, a western blot experiment was carried out in parallel with the Far-western to compare the result of the to experiments. In a normal Western-blot, no additional bands should appear as in Far-western at 50 kDa, because in normal western-blot, the purified protein is not allowed to bind to the blotted proteins, which could be as pspA candidate**.** The signals at 40 and 27 kDa in both blots are unspecific. It turned out that pspA has one target protein in *B. subtilis* as in *E. coli.*

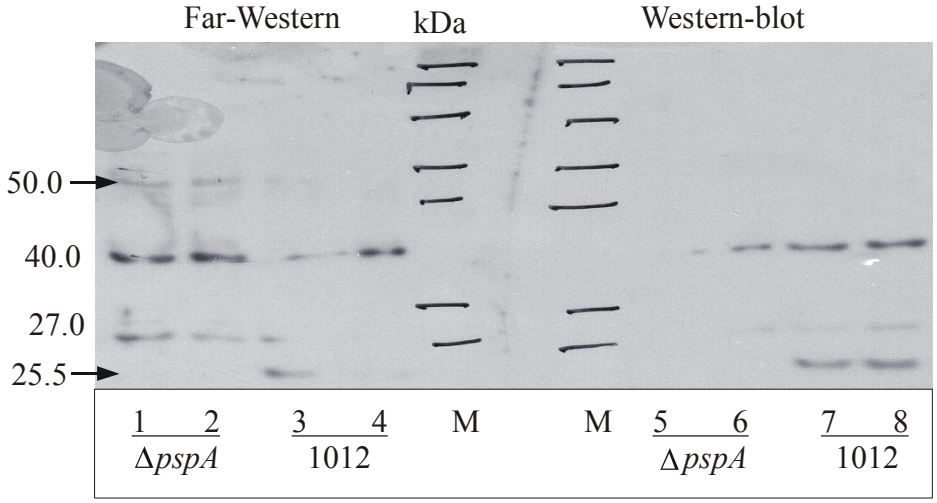


Fig. 21: Far-Western and Western-blot analysis of the PspA protein. Far-Western and Western-blots of cell extracts from the strain AA03 (Δ*pspA*) (lanes 1, 2, 5 and 6) and *B. subtilis* strain 1012 (lanes 3, 4, 7 and 8). The signals at 25.5 kDa (PspA) and at 50 kDa (PspA partner) are marked by arrows. The bands at 40 and 27 kDa are unspecific. M, molecular weight marker fragments.

4.4 Construction and analysis of transcriptional fusions using the *lacZ* reporter gene

Besides the Northern-blot analysis, the construction and analysis of transcriptional fusions has been turned out to be a fast and efficient method to study gene regulation. The transcriptional fusions to *lacZ* have already been constructed to about 2000 genes of unknown function, so-called y-genes (Schumann *et al*., 2001). Therefore, I decided to make use of some of these fusions, and to measure the β-galactosidase activity before and after alkali treatment of the cells. Transcriptional fusions to the following genes have been tested: *ykoY*, *pstBA, hisI, ycgM, yxbB*, and *yybp*. It turned out that only the genes *ykoY* and *pstBA* exhibited a significant increase after alkali challenge (see below). While *ykoY*::*lacZ* was induced more then 20-fold, *pstBA*::*lacZ* was induced more than 60-fold. Since *pstBA* belongs to the pentacistronic *pst* operon, another gene from this operon was tested namely *pstS*::*lacZ*. This gene, too, turned out to be alkali-inducible (about 40-fold). Therefore, I asked whether addition of phosphate to the medium cause induction of the *pst* operon.

4.4.1 Transcriptional analysis of the *ykoY* gene

The β-galactosidase activities exhibited by strain BSF1847 carrying the *ykoY*::*lacZ* operon fusion is shown in Fig. 22. This fusion becomes induced between 0 and 15 min after alkali challenge about 16-fold. To confirm this result obtained by the enzymatic assay, a Northern blot was carried out using a *ykoY* antisense probe (Fig. 23) and RNA prepared from strain 1012. This experiment led to the detection of a transcript of about 1 kb which by length corresponds to the *ykoY* gene (971 bp). It became strongly induced within the first 10 min after alkali stress and declined thereafter. From the genomic analysis, *ykoY* should form a bicistronic operon with *ykoX*. But this result is in accordance with those obtained by
DNA macroarray analysis where only *ykoY* has been found to be induced by alkali shock (Wiegert *et al*., 2001). To confirm this result, ß-galactosidase activity was measured using strain BSF1847 carrying the *ykoX* ::*lacZ* fusion. It turned out that this fusion exhibited no induction after addition of alkali (data not shown).

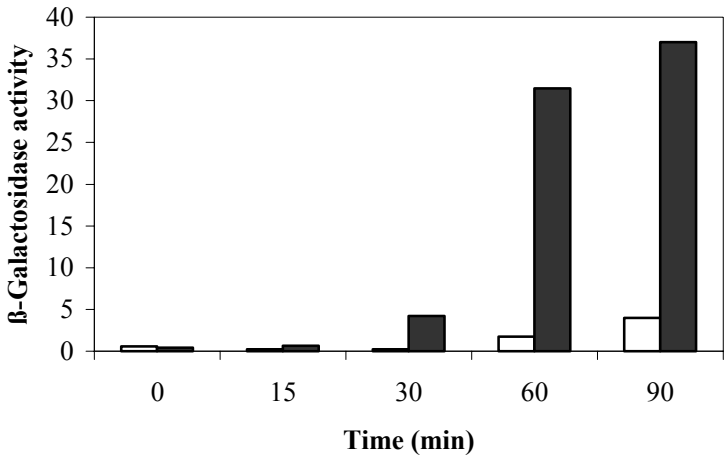


Fig. 22: Transcriptional analyses of the *ykoY* gene. Cell were grown in LB medium at pH 7.4 to mid-exponential phase, divided into two halves where one half was further incubated at the neutral pH, while the secound half was alkali-induced by the addition of 2 N NaOH to rise the value to 8.9. Aliquots were taken before (white bars) and after addition of NaOH (black bars) at different time points as indicated. The strain AA08 (*ykoY::lacZ*) was used to measure the ß-galactosidase activity. The activities are given in Miller units.

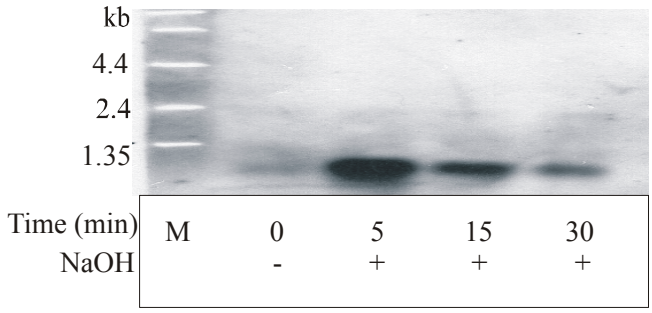


Fig. 23: Transcriptional analysis of the *ykoY* gene. Northern blot of total RNA extracted from *B. subtilis* 1012 wild-type, before (-) and 5, 15 and 30 min after addition of NaOH (+). A *ykoY*-specific DIG-labelled antisense RNA riboprobe was used for hybridisation. M, molecular weight marker fragments.

In addition, a primer-extension experiment was carried out to map the potential transcription start point. One single band could be detected which increased after alkali shock corresponding to a T in the transcript (Fig. 24).

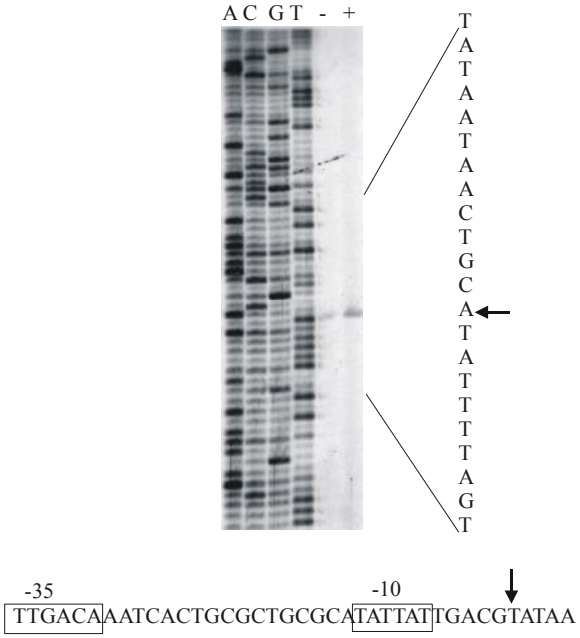


Fig. 24: Mapping of the *ykoY* promoter. The 5` end of the *ykoY* transcript was mapped by primer extension. Equal amounts of total RNA isolated from *B. subtilis* 1012 before and 20 min after alkali shock were used. The potential transcriptional start point is marked by arrows. Lanes A, C, G, and T show the dideoxy sequencing ladder obtained with the same oligonucleotide used for the primer extension reaction.

>>>>>

aatatatgcttccgatcgggatagcgattttggcgctg**ttcct**gctctacttactgatga

<<<<<

taaag**aggaa**gaaacgcacgcactccgaacaattatctgtatttttgaaaaataaacg

>>>>> >>>>> >>>>> >>>>> >>>>>

TTGACAaatcactgcgctgcgcaTATTATtgacg**T**ata**aaatc**atatttc

>>>>>

ttg**aaatc**ccaaaggggagtagcgtccgga**ATG**tattccggaaacaaagtcgtca

Fig. 25: Representation of the DNA-sequence upstream of *ykoY*. In the sequence, the transcription start point is marked by arrow. (>>> <<<<) refers to indirect repeats, while (>>> >>>) to refers direct repeats. The -10 and –35 promoter boxes are marked by red.

4.4.2 Transcriptional analysis of the *pst* operon

As shown under Fig. 26 A and B, *pstS::lacZ* and *pstBA::lacZ* are alkali-inducible.

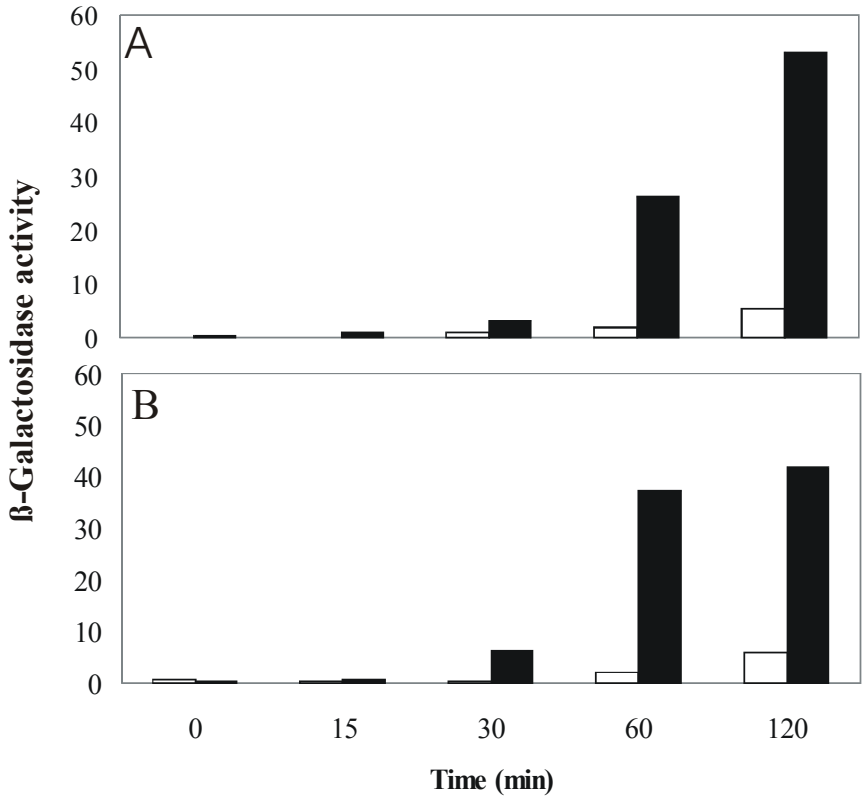


Fig. 26 : Transcriptional analyses of the *pst* operon. Cell were grown in LB medium at pH 7.4 to mid-exponential phase, divided into two halves where one half was further incubated at the neutral pH, while the second half was alkali-induced by the addition of 2 N NaOH to rise the value to 8.9. The strains *pstS::lacZ* and *pstBA::lacZ* were used to measure the ß-galactosidase activity at different time points before (white bars) and after alkali shock (black bars). The enzyme activity of both fusions namely Strains AA04 *pstS::lacZ* (A) and AA05 *pstBA::lacZ* (B). The activities are given in Miller units.

These data suggest that all genes of the *pst* operon are alkali-inducible. To prove this assumption, a Northern-blot experiment was carried out. Total RNA was prepared before and at different times after alkali challenge, subjected to a Northern blot and probed with *pstBA* antisense RNA. While no signal was obtained in the absence of alkali shock, a signal started to appear 5 min after induction and increased at least up to 60 min (Fig. 27). The size of this signal was determined to be about 4.4 kb which is in good agreement with the length of the *pst* operon (4.4 kb). This result exhibits that the complete *pst* operon is induced by an alkali shock.

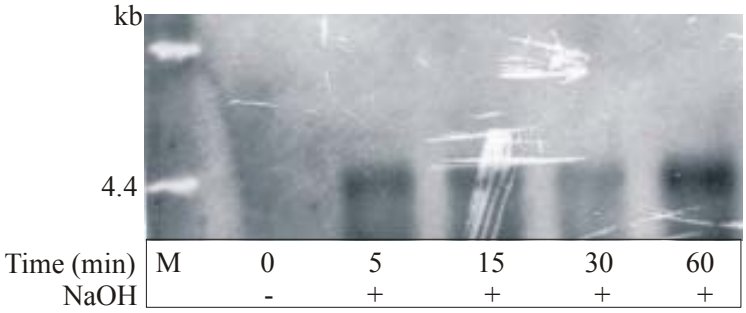


Fig. 27: Transcriptional analysis of the *pstBA* gene. Northern blot of total RNA extracted from *B. subtilis* 1012 before and 5, 15, 30 and 60 min after addition of NaOH. A *pstBA*-specific DIG-labelled antisense RNA riboprobe was used for hybridisation. M, molecular weight marker fragments.

Is the transcription start point used after alkali shock identical to the one determined after phosphate starvation (Qi *et al*., 1997) or is a different one used? To answer this question, a primer-extension experiment was carried out. Total RNA prepared before and 15 min after alkali challenge was reverse transcribed, and the same oligonucleotide was also used to prime the DNA sequencing reactions. While no signal was obtained in the absence of alkali shock, a strong one became visible after alkali challenge (Fig. 28). This signal corresponds to a "C" residue in the codogenic strand and is identical to the transcription start site determined after phosphate starvation (Qi *et al*., 1997). Therefore, the same transcriptional start site is used both after alkali stress and phosphate starvation suggesting that the alkali shock mimics phosphate starvation.

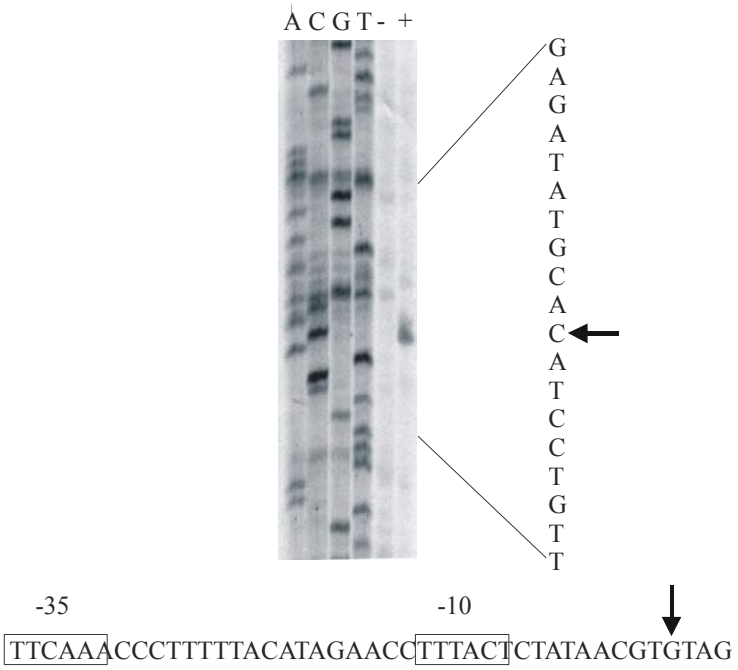


Fig. 28: Mapping pf the *pstBA* promoter. The 5` end of the *pstBA* transcript was mapped by primer extension. Equal amounts of total RNA isolated from *B. subtilis* 1012 before and 20 min after alkali shock were used. The potential transcriptional start point is marked by an arrow. Lanes A, C, G, and T show the dideoxy sequencing ladder obtained with the same oligonucleotide as used for the primer extension reaction.

4.4.3 Alkali-induction of the *pst* operon depends on the PhoP-PhoR two-component system

Expression of the *pst* operon is under positive control of the PhoPR two-component signal transduction system (Qi *et al*., 1997). Therefore, this finding prompted to investigate whether alkali-induction of this operon is also dependent on the Pho system. A *phoR*::*tet* allele (*phoR* codes for the sensor kinase) was crossed into the *pstBA*::*lacZ* background, and the resulting strain AA07 was analysed for the β-galactosidase activity in the absence and presence of alkali. It turned out that in the absence of the sensor kinase, the enzymatic activity did not increase (Fig. 29). This result suggests that the PhoPR two-component signal transduction system is necessary for the induction of the *pst* operon.

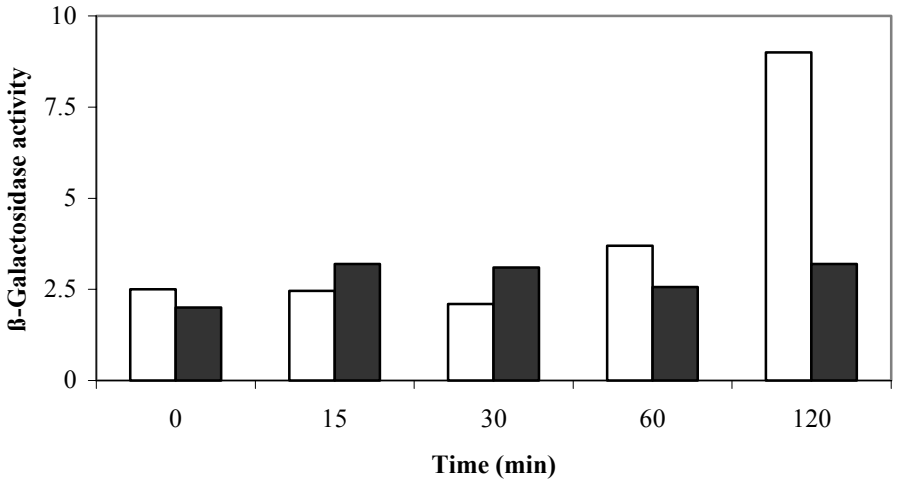


Fig. 29: The induction of the *pst* operon is dependent on the two-component Pho system. Cell were grown in LB medium at pH 7.4 to mid-exponential phase, divided into two halves where one half was further incubated at the neutral pH, while the second half was alkali-induced by the addition of 2 N NaOH to rise the value to 8.9. The strain AA07 carrying *phoR*::*tet* allele and *pstBA*::*lacZ* was used to measure the ß-galactosidase activity at different time points before (white bars) and after alkali shock (black bars). The activities are given in Miller units.

4.4.4 Alkali-induction is specific for the *pst* operon

To find out whether this behaviour is specific for the *pst* operon or affects all members of the Pho regulon, the *phoA*::*lacZ* fusion (strain AA06) was analysed. This fusion did not respond to an alkali shock (Fig. 30). To summarize the *pst* operon was induced by an alkali challenge, while expression of the *phoA* gene was not influenced at all by the external pH. This result show that this induction behaviour is specific for the *pst* operon and does not affect other members of the Pho regulon. This conclusion is also supported by the DNA macroarra experiments which failed to detect induction of other members of this regulon (data not shown)

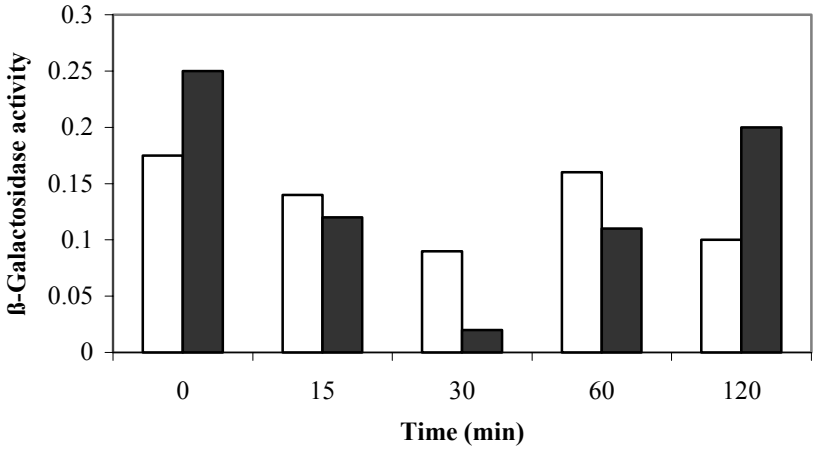


Fig. 30: Alkali induction is specific for the *pst* operon. Cell were grown in LB medium at pH 7.4 to mid-exponential phase, divided into two halves where one half was further incubated at the neutral pH, while the second half was alkali-induced by the addition of 2 N NaOH to rise the value to 8.9. The strain (AA06) carrying the strain *phoA*::*lacZ* was used to measure the ß-galactosidase activity at different time points before (white bars) and after alkali shock (black bars). The activities are given in Miller units.

4.4.5 Addition of phosphate prevents alkali-induction of the *pst* operon

Since data reported above have been shown that an alkali shock mimics phosphate starvation to the *B. subtilis* cells, next I explored whether increasing the phosphate concentration within the LB medium could prevent alkali induction of the *pst* operon. The *B. subtilis* strains AA04 and AA05 carrying the *pstS*::*lacZ* and *pstBA*:*lacZ* fusions respectively were grown in LB medium with added phosphate to 1 mM, and the β-galactosidase activity was measured in the absence and after alkali-induction. It turned out that indeed the higher phosphate concentration within the medium completely prevented induction of the two operon fusions after increasing the external pH (Fig. 31 A, B). These results suggest that the alkali shock as carried out in these experiments signals phosphate starvation to the cells which can be overcome by the addition of phosphate.

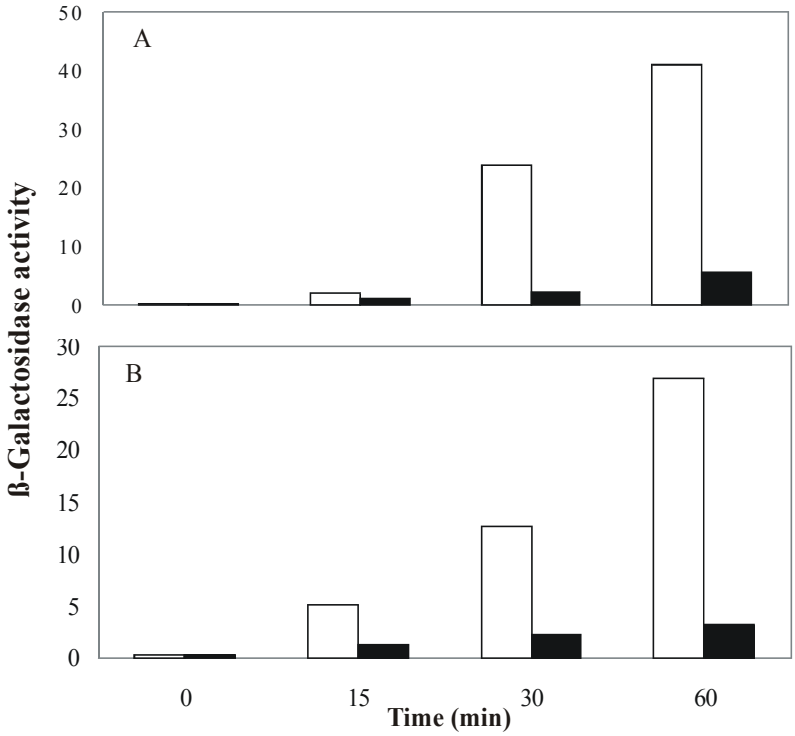


Fig. 31: Addition of phosphate prevents alkali-induction of the *pst* operon. The *B. subtilis* strains AA04 *pstS*::*lacZ* (A) and AA05 *pstBA*:*lacZ* (B) were grown in LB medium with added phosphate to 1 mM, and the β-galactosidase activity was measured at time points indicated in the present (white bars) and in the absence (black bars) of alkali.

4.4.6 An alkali shock reduces uptake of phosphate

What could be the explanation for the finding that an alkaline pH within the medium signals phosphate starvation conditions to the cells? One possibility could be that the alkaline pH directly influences the activity of a low-affinity phosphate uptake system. To investigate whether such a low-affinity phosphate uptake system is indeed affected by the external pH, phosphate uptake was measured in the absence and presence of alkali challenge. The results presented in Fig. 32 A, B demonstrate a rapid uptake of ^{32}P at the neutral pH, whereas the uptake was completed abolished at an external pH of 8.9.

By similarity with known low-phosphate transport systems, the *pit* gene has been suggested to code for such a system in *B. subtilis* (http://genolist.pasteur.fr/SubtiList). To investigate whether the external pH of 8.9 can be affected by the low-affinity phosphate uptake system of the strain AA13 (*pit*::pMUTIN), a phosphate uptake was measured in the absence or in presence of alkali challenge.

These data are in agreement with the assumption that the external alkaline pH might affect a low-affinity phosphate uptake system which can be overcome by increasing the phosphate concentration within the medium (Atalla A. 2003).

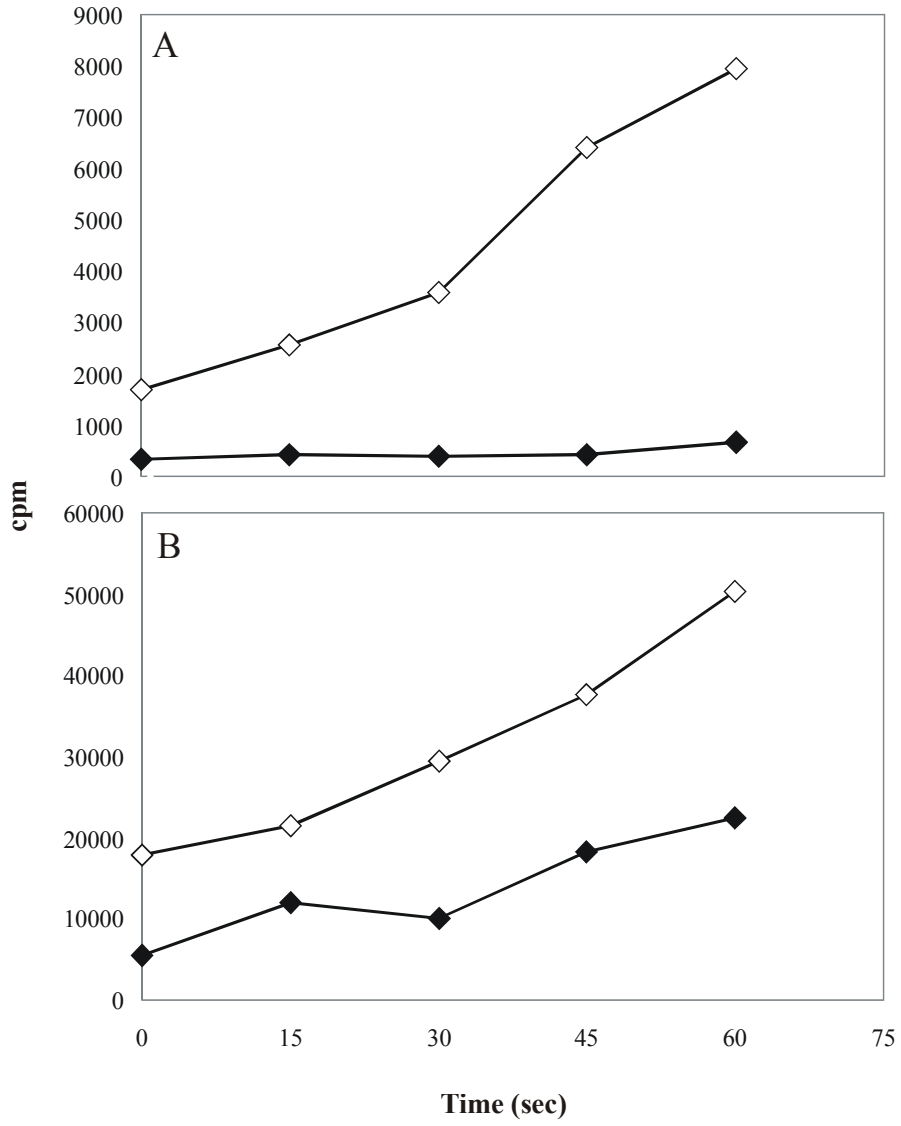


Fig. 32: An alkaline pH in the growth medium prevents uptake of added radioactive phosphate. Uptake of radioactive phosphate (^{32}P) was measured essentially as described by Prágai *et al*. (2001). Strains were grown in low phosphate medium (LPM) to the stationary phase. Then, cells were harvested by centrifugation, washed twice in LPM without phosphate, resuspended in the original volume of 20 ml, divided into to two halves and further shaken to an OD_{578} of 0.7 to induce for maximal phosphate uptake. Then, 995 µl cells were mixed with 5 µl of ^{32}P (185 MBq) in a final concentration of 10 µM P_i. Probes were taken at 15 sec intervals and the amount of radioactive phosphate taken up by the cells was measured. (A) 1012 strain (B); strain AA13 (*pit*::pMUTIN).

5 Discussion

Bacteria are equipped with numerous genetic programs allowing them to adapt to diverse stressful conditions such as sudden changes in temperature (heat and cold shock) and external pH (alkaline and acid stress), oxidative and hyperosmotic stress, and severe DNA damage (see the book edited by Storz and Hengge-Aronis, 2000, for a recent and updated review on this subject). As outlined in detail at the beginning of the introductory part, bacteria have developed a stress response pathway where a sensor recognises the stress factor either directly or indirectly through the production of a substance which is produced under the influence of the stress factor (e.g., heat shock leads to the production of denatured proteins in the cytoplasm sensed by the DnaK chaperone in *E. coli* or the GroE chaperone in *B. subtilis*; (Yura *et al*., 2000; Schumann, 2003). The activated sensor interacts directly or indirectly with a transcriptional regulator, either an alternative sigma factor, a transcriptional activator or repressor. This in turn leads to the induction of a subset of genes the products of which cope with the stressful situation to allow survival and even further growth. This regulatory pathway also ensures return to the prestimulus state by a feedback loop (see Fig. 1). All stress response pathways studied result in the induction of the stress genes at the level of transcription.

Elucidation of a given stress response pathway is carried out in most cases by first identifying stress genes by virtue of their increased transcription rate following application of the stress factor. An alternative approach is to search for orthologs in the new organism, orthologs of genes where is has been shown that they are induced by that stress factor in another bacterial species (Wetzstein *et al*., 1992; Schmidt *et al*., 1992). Induced expression following exposure to a stress factor has been monitored in the past by at random fusion of genes to a reporter gene such as *lacZ* (Völker *et al*., 1993), and nowadays by analysis of the complete transcriptom using either DNA micro- or macroarrays (Price *et al*., 2001; Wiegert *et al*., 2001; Mäder *et al*., 2002b). If most or all stress genes of a given stimulon have been identified, the next step is to look for the potential regulator(s). In all cases studied so far, the gene coding for the regulator is part of the stress stimulon being involved in autoregulation and either an alternative sigma factor or a DNA-binding protein. Both can be identified by specific signatures (sigma factors contain two, DNA-binding proteins one helix-turn-helix motif). Therefore, the transcriptional regulator of a given stress regulon or regulators of the stimulon should be member(s) of identified stress genes.

The present doctoral thesis deals with the identification of transcriptional regulators of the alkali stress stimulon of *B. subtilis*. The alkali shock is a not well-studied stress response. In *E. coli*, several genes have been identified, mainly by the work of the group of J. Slonczewski (see Sloncziewski and Foster, 1996, for a recent review) though their regulation remains elusive. In *B. subtilis*, a transcriptom analysis has revealed the presence of about 80 genes induced at least four-fold upon alkali challenge which can be divided into three classes (Wiegert *et al*., 2001). The first class contains genes that are the under control of the alternative sigma factor σ^W (Huang *et al*., 1997; 1999). The second class contains genes which are induced by alkali stress in a σ^W-dependent manner but which have not been confirmed to belong to the σ^W regulon so far. The function of most of these genes is unknown. It is possible that some proteins are linked to an extracytoplasmic function, for example the ABC transporters (Wiegert *et al*., 2001). The last group contains the genes which are clearly independent of σ^W. Expression of some of these genes is partially reduced in the *sigW* knockout, suggesting that the regulation of these genes is more complex and that additional mechanism(s) are involved (Wiegert *et al*., 2001). The genes with an expression not clearly dependent on σ^W are summarized in Tab. 1

The aim of the present work was to identify one or more transcriptional regulator involved in the regulation of alkali-inducible genes not being member of the σ^W regulon using two different experimental strategies: (1) construction and analysis of knockouts coding for alkali-inducible regulatory proteins, and (2) DNA macroarray technology. In addition, alkali-induction of the *pst* operon was studied, and the regulator could be identified.

5.1 Construction and analysis of knockouts within the three potential regulatory genes *kipR*, *yvdT* and *pspA*

The genes *kipR*, *yvdT* and *pspA* code for potential transcription regulators and are induced after raising the external pH value in the medium. Therefore, it can be assumed that they exert a regulatory function during adaptation of the cells to the alkaline shock. Therefore, I first asked whether *B. subtilis* cells carrying knockouts in these three genes will exhibit a phenotype. It turned out that growth in the absence of these three proteins at both the neutral pH of 7.4 and the alkaline pH of 8.9 occurred unimpaired (Fig. 6). When expression of some selected alkali-inducible genes not belonging to the σ^W regulon was analysed in these

knockouts by Northern blotting, no significant difference was found before and after alkali induction suggesting that expression of these genes is not regulated by any one of these three regulatory proteins.

The *kipR* gene has been identified as the sixth gene of the potential heptacistronic operon *ycsF-ycsG-ycsI-kipI-kipA-kipR-ycsK* (Wang *et al*., 1997). *The kipR* operon induced by glucose when readily available source of nitrogen, such as glutamine or ammonia, are scarces. This operon is regulated by TnrA activation and by KipR repression (Wray *et al.,* 1996).

The TnrA positively regulates a number of genes and operon coding for proteins that degrade nitrogen-containing compounds (Ferson et al., 1996; Wray et al., 1996).

In addition, the TnrA-dependent promoter are characterized by a common upstream sequence (TGT*NAN*$_7$T*NA*CA), two of them are present in the promoter for the *kip* gene-containing operon. The nitrogen-limited TnrA activation is clearly antagonized by KipR repression (wang *et al*., 1997).

The sixth gene of the *kipR* operon is *ycsO*, now renamed *kipR*, codes for a protein of 247 amino acids whose sequence contains a helix-turn-helix motif typical of DNA-binding proteins. The *kipR* is similar to transcriptional regulator (IclR family). In addition to KipR regulator, the transcriptional regulator PcaU from *Acinetobacter* sp. is a member of the IclR protein family (Popp *et al.,* 2002).

The transcriptional switch on of ssgA by A-factor, which is essential for spore septum formation in Streptomyces griseus is appeared to regulate by the SsfR , which is encodes an IclR-type transcriptional regulator (Yamazakai *et al.,* 2003).

Deletion of *kipR* resulted in an about 10-fold increase in transcription at the promoter located in front of the whole operon (Wang *et al*., 1997). Therefore, this gene codes for a transcriptional repressor protein at least under the conditions tested. But many transcriptional regulators have a dual function being active either as a repressor or as an activator depending on the location of the binding site.

In addition, the potential regulator YvdT is a member of the TetR family. To this protein family belongs the local repressor AcrA, which plays a modulating role in the regulation of acrAB genes of *E. coli* by global stress signal (Ma D et al., 1996).

5.2 Identification of PspA as a transcriptional anti-activator

The *pspA* gene codes for a novel class of transcriptional regulator protein called anti-activator. These proteins exert there function by interacting with their cognate activator protein thereby preventing its binding to the DNA. One example for such an anti-activator is the Aes protein of *E. coli* which sequesters the MalT transcriptional activator (Joly *et al*., 2002). A second example is the PspA protein which has been suggested to bind to the transcriptional activator protein PspF based on genetic data (Jovanovic *et al*., 1999). Later, it could be shown by *in vitro* experiments using purified proteins that PspA and PspF form a complex (Elderkin *et al*., 2002). PspA negatively controls expression of the *psp* operon in *E. coli*, and this operon is induced by different stress regimen including alkali stress (Weiner and Model, 1994). In addition, PspA exerts a second function by playing a general role in maintaining the integrity of the inner membrane (Kleerebezem *et al*., 1996).

A *pspA* orthologue has been identified in *B. subtilis* first designated as *pspB* (Graumann *et al*., 1996) and later as *pspA* (Kunst *et al*., 1997), but no *pspF* orthologue. And the *B. subtilis pspA* turned out to be alkali-inducible as well and being member of the σ^W regulon (Wiegert *et al*., 2001). These data suggested that *pspA* controls expression of at least one operon which is turned off after alkali shock by sequestering a so far unknown transcriptional activator. Experiments have been carried out to identify this transcriptional activator and genes under indirect negative control of the *pspA*. Since polyclonal antibodies have been raised against purified His-tagged PspA, the Far-Western blot technique has been chosen to identify the potential partner protein(s) of PspA. When proteins prepared from a *pspA* knockout and separated by SDS-PAGE were first incubated and then probed with the purified PspA antibodies, one single band of about 50 kDa could be detected. Attempts to purify this complex after immunoprecipitation failed. Therefore, a different method has to be tried to obtain sufficient amounts of this protein to allow its identification by MALDI-TOF-MS. One alternative would involve immobilization of PspA on a solid phase followed by addition of a cell extract under conditions where the partner protein would bind to PspA. After extensive washing, the partner protein has to be eluted by using an appropriate buffer.

The rationale to identify genes being under the indirect negative control of *pspA* was based on the assumption that high amounts of PspA should result in decreased expression of these

target genes. Enhanced expression of *pspA* was obtained by fusing the gene to an IPTG-inducible promoter. Total RNA was isolated under conditions of overexpression of *pspA* and individual transcripts were quantified using a DNA macroarray containing PCR products of all protein-coding *B. subtilis* genes. It turned out that 14 genes were downregulated by a factor of at least three as compared to the control RNA preparation (Table 7). These genes are good candidates for being under the indirect negative control of *pspA*. This artificial overexpression mimics alkali induction of *pspA* at neutral pH though, at least at the level of *pspA* transcript, addition of NaOH led to a higher induction of *pspA* as compared to the induction with IPTG (see Fig. 15). If the genes listed in Table 7 are indeed under the positive control by a transcriptional activator a common binding site should be located upstream of promoter sequence. Inspection of the DNA sequences around the promoter of these genes revealed that six genes, which are downregulated under the conditions of the PspA overexpression have similar sequences in the direct and indirect repeats (see Fig. 18). To identify the promoter of these genes, besides the *cotS* which is dependent on σ^K factor, no known prompter sequence could be identified. It indicates that these genes are under the control by so far unknown sigma factor.

Besides *E. coli* and *B. subtilis*, *pspA* orthologues have been described in Yersinia enterocolitica (Darwin and Miller, 2001) and in pea chloroplasts (Li *et al*., 1994). The PspF transcriptional activator belongs to the enhancer binding protein (EBP) family (for a recent review, see Studholme and Dixon, 2003) which interact with the σ^{54} subunit of the RNA polymerase holoenzyme ($E\sigma^{54}$). These transcriptional activators are usually bound at least 100 bp upstream of the promoter site, and DNA looping is required for the activator to contact $E\sigma^{54}$. In the case of the *psp* operon of *E. coli*, this looping is stimulated by binding of the integration host factor (IHF). These activators consist normally of three functional domains, a DNA-binding domain containing a helix-turn-helix sequence motif, a σ^{54} interaction module and a regulatory input domain. This regulatory input domain is absent from PspF (Jovanovic *et al*., 1996); instead, its activity is controlled by formation of a repressive complex with the PspA protein. A model displaying activation of $E\sigma^{54}$ bound upstream of the *psp* operon is presented in Fig. 33.

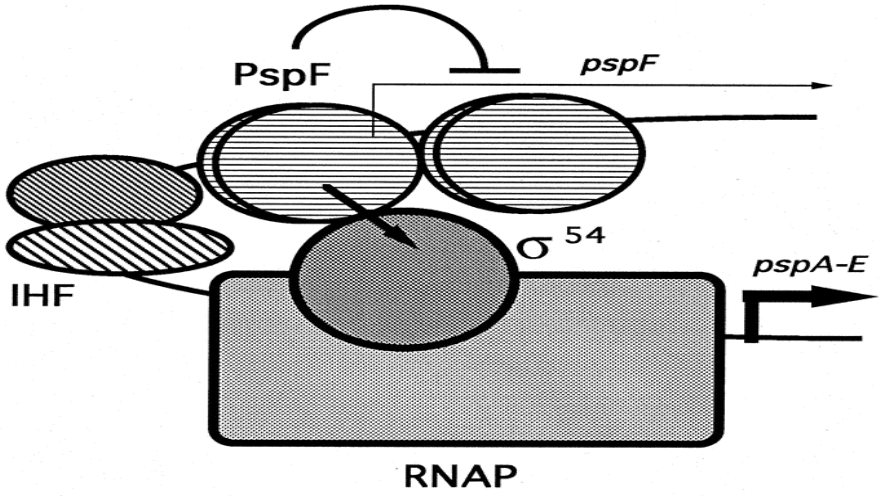


Fig. 33: Model for activation of the *psp* operon transcription by the PspF activator under inducing conditions. IHF binds between the PspF and PspA σ^{54}-dependent promoter and bends the DNA while PspF binds to the upstream-activating-sequence (UAS) and interacts with the σ^{54}-RNA polymerase complex (adapted from Jovanovic and Model, 1997).

5.3 Analysis of the *pst* operon

The DNA macroarray analysis has revealed that the genes of the *pst* operon are alkali-inducible (Wiegert *et al*., 2001). This operon is member of the PhoP-PhoR two-component signal transduction system, which controls the phosphate deficiency response in *B. subtilis* (for a review see Hulett, 1996). When cells are starved for phosphate, several genes are either activated or repressed by the phosphorylated response regulator among them the *pst* operon and the *phoA* gene (Liu and Hulett, 1998; Qi *et al*., 1997). While the *pst* operon codes for a high affinity phosphate transport system (Qi *et al*., 1997), the *phoA* gene encodes an alkaline phosphatase (Hulett *et al*., 1991). The objective of this part of the study was to find out why the *pst* operon is alkali-inducible and not the other genes of that regulon and to identify the regulator involved.

To confirm the results obtained by the DNA macroarray technique by an independent experimental approach, transcriptional fusions between *pstS* and *pstBA* and the *lacZ* reporter gene were analysed. Both operon fusions turned out to be alkali-inducible. Next, the Northern-blot technique was used to show that the whole *pst* operon is alkali-inducible. In the

third experiment, the transcriptional start point was determined and shown to be identical to that found during phosphate starvation (Qi *et al*., 1997). Therefore, the same transcription start site is used both after alkali stress and phosphate starvation suggesting that the alkali shock mimics phosphate starvation. Since the *pst* operon is under the positive control by the PhoP-PhoR two-component system as already mentioned, I asked whether this regulatory system is also needed to induce the *pst* operon during alkali stress. When induction of the *pstBA*::*lacZ* fusion was measured in the presence of the *phoR*::*tet* null mutation, no increase of the β-galactosidase activity was found. This finding allowed the conclusion that the two-component Pho system is indeed involved in the alkali-induction of the *pst* operon.

Based on the interpretation that an alkali shock mimics phosphate starvation to the *B. subtilis* cells, I explored whether increasing the phosphate concentration within the medium could prevent alkali induction of the *pst* operon. This turned out to be the case. What could be the explanation for the finding that an alkaline pH within the medium signals phosphate starvation conditions to the cells? One possibility could be that the alkaline pH directly influences the activity of a low-affinity phosphate uptake system. Measurement of ^{32}P uptake revealed that a rapid uptake occurred at neutral pH, and this was completely abolished at an external alkaline pH of 8.9. These data are in agreement with the assumption that the external pH might affect a low-affinity phosphate uptake system which can be overcome be increasing the phosphate concentration within the medium. By similarity with known low-phosphate transport systems, the *pit* gene has been suggested to code for such a system in *B. subtilis* (http://genolist.pasteur.fr/SubtiList). Uptake of ^{32}P was also measured in a *pit* knockout *pit*::pMUTIN.

E. coli codes for two major P_i transport systems (Nakata *et al*., 1987; Rosenberg *et al*., 1987; 1977; Surin *et al*., 1987; Webb *et al.,* 1994). The Pst (phosphate-specific transport) system is a high-affinity, low-velocity, free-P_i transport system which is structurally similar to ABC transporters (Ames *et al*., 1986). The Pit (phosphate inorganic transport) system is a divalent metal transporter of which Pi (Van Veen *et al*., 1994) or arsenate (Willsky *et al.,* 1980) can serve as the anion (Van Veen *et al*., 1994). The Pst and Pit are two systems that actively transport inorganic phosphate (P_i) into *E. coli* cells. PstS is a periplasmic P_i-binding protein, PstC and PstA are integral membrane proteins that mediate the translocation of P_i through the inner membrane (Webb *et al*., 1992) and PstBA is an ATPase that energizes the transport (Chan and Torriani *et al*., 1996). The function of PstBB is not yet clear. The *pst* operon has a

role in the regulation of Pho genes (Amemura *et al*., 1982; Wanner *et al*., 1980; Willsky *et al*., 1973) in that *phoU* is required for the repression of the Pho regulon but is apparently not required for phosphate transport through the Pst system (Nakata *et al*., 1984; Steed *et al*., 1993; Zuckier *et al.,* 1981). In *B. subtilis*, Pho regulon genes are expressed in response to phosphate starvation and regulated by the PhoP-PhoR two-component system. Previous data showed that PhoP and PhoR are equally required for transcription induction of Pho regulon genes, including *phoA*, *phoB* and *phoPR* (Hulett *et al*., 1994a,b; 1995; 1996).

5.4 Outlook

At the moment, it is still unclear whether *kipR* and *yvdT* are involved in the regulation of alkali-inducible and –repressible genes. In general, one would assume that a gene coding for a repressor protein and induced after alkali shock would be involved in the turn-off of genes under these adverse conditions. On the other hand, an alkali-inducible regulatory gene involved in the transcriptional activation of target genes should lead to the enhanced transcription of these genes. If transcription of these target genes is analysed in knockouts, genes under negative control will fail to be shut-off while those under negative control will fail to be turned on. Whether *kipR* and *yvdT* code for alkali-inducible repressors or activators is unknown. There is only one report dealing with *kipR* where it has been described that it codes for a transcriptional repressor (Wang *et al*., 1997), while the role of *yvdT* has never been analysed. Even if KipR has been identified as a repressor of its own operon, it cannot be concluded that it also acts as a negative regulator since many proteins act as dual regulators. One prominent example is the AraC protein of *E. coli* which acts as a repressor of the *araBAD* operon in the absence of a arabinose and as an activator in the presence of the sugar (Schleif, 2000).

To find out whether *kipR* and *yvdT* act either as a repressor or activator, one could follow a global strategy. This means to prepare anti-sense RNA from all alkali-inducible (not belonging to the σ^W regulon) and alkali-repressed genes and to use them to probe total RNA preparations from the *kipR* and *yvdT* knockouts at neutral and alkaline pH. Alternatively, though more costly, these RNA preparations could be first reverse transcribed into ^{33}P-labelled cDNA and then used in a hybridisation experiment with a DNA macroarray. In the case of a repressor, alkali-inducible genes would fail to become repressed in the knockout, and in the case of an activator they will fail to become induced.

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Abbreviations

A	Adenine
A_{260}	Absorption at a wavelength of 260nm
A_{280}	Absorption at a wavelength of 280nm
Ap	Ampicillin
(APS)	Ammoniumperoxodisulfate
ATP	Adenosin-5`-triphosphat
bp	Base pairs
B. subtilis	*Bacilus subtilis*
C	Cytosin
^{O}C	Degrees Centigrade
cat	Gen of chloraphenicol-acetytransferase
Cm	Chloramphenicol
dd	Dideoxyribose
DEPC	Diethylpyrocarbonate
E. coli	*Escherichia coli*
Em	Erythromycin
et al.	Et alteri
G	Guanine
g	Gram
h	hour
His	Histidin
Hsp	Heat-shock protein
IAA	Isoamylalkohol
IPTG	Isopropyl-ß-D-thiogalaktosid
kb	Kilobase
kDa	kilo-Dalton
l	liter
LB	Luria-Bertani (growth medium)
LPM	Low-phosphate medium
M	Molar
µg	Microgramm
µl	Microliter
MG	Molecolare weigth
Mg	Milligramm
min	Minute
ml	mililiter
mM	milimolar
MOPS	Morpholiopropansulfon acid
mRNA	messenger-RNA
MW	molekular weight
Neo	Neomycin
NGS	Normal –Goat-Serum
nm	Nanometer
OD_{578}	Optical Density at a wavelength of 578 nm
^{32}P	Isotope phosphor
PAGE	Polyacrylamide Gel electrophoresis
PCR	Polymerease Chain Reaction
pmol	Picomol
PBS	Phosphate-buffer saline

PVDF	polyvinylidene difluoride
rpm	Revolutions per minute
RT	Room temperature
S cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium Dodecylsulphate
Sec.	Second
Tab.	Table
TEMED	N,N,N`,N`-Tetramethylenethylendiamide
Tris	Tri-(hydroxymethyl)-aminomethane
Tween-20	Polyoxyethylensorbitane Monlaurate
U	Units (enzym activity)
UV	Ultra-violet
v/v	Volume/Volume
w/v	Weight/Volume
Δ	Deletion

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Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Disseration einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschulen endgültig nicht bestanden.

Bayreuth, 05.05.2003 Atalla, Akram