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STRESS RELATED CHANGES OF CELL SURFACE HYDROPHILICITY IN *BACILLUS SUBTILIS*

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The changes of cell surface hydrophilicity in *Bacillus subtilis* were analyzed in response to oxygen-limitation, heat shock, salt stress, pH-shock, phosphate- and carbon-limitation. Although cell surface hydrophilicity varied during growth phases, an increase of surface hydrophilicity was observed under several of these stress conditions. An observed drop in intracellular GTP and/or ATP may be an element of the signal transduction pathway leading to an increase in surface hydrophilicity in response to environmental stresses. Attachment of cells to soil particles under salt stress conditions is strongly influenced by the *degS/degU* two-component system, which thereby provides a mechanism for the bacteria to escape from the hostile environment.

Keywords: *Bacillus subtilis*, cell surface, stress, *degS/degU*

Introduction

Bacteria in their natural environment spend most of their lives under growth limiting conditions, which are associated with a variety of stresses. Understanding of the strategies used to cope with stress conditions is important for both basic microbiology and applied biotechnology.

Bacillus subtilis, living in the upper-layer of the soil, is affected by many kinds of stresses (phosphate-, carbon-, energy-, and oxygen-limitation, osmotic stress, heat- or cold shock, etc.). As they live attached to the solid soil particles, the adhesion

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features of the *B. subtilis* cells play a crucial role in their life cycle and in their stress-tolerance.

The exact physiological role and significance of many stress gene products is not yet fully understood. To establish the role of stress proteins in the adaptational behavior of cells, the molecular approach is an indispensable tool. Several recent publications deal with this aspect [1, 2, 3].

The data presented in this paper suggest that certain stress-induced metabolic responses may participate in forming a cell surface level response (CSLR): the change of the hydrophilicity of the cell surface during stress response. In the cases of phosphate-limitation, salt stress, phosphate- and carbon-starvation and the entering of exponentially growing bacteria into the stationary phase, the surface of the *B. subtilis* cell becomes more hydrophilic. This phenomenon is markedly different from that of Gram negative bacteria as described in *Salmonella typhimurium* [4]. In a related study, both chemical treatments and changes in incubation temperature influenced cell surface hydrophobicity and cell surface charge in *Azospirillum* spp. [5] but, unfortunately, no kinetic analysis was carried out.

The aim of the work presented here is to provide kinetic data about CSLR, to investigate the behaviour of potential signal transduction pathway elements and to elucidate the role of the DegS/DegU system in the development of CSLR and in the escape mechanism of the bacteria from an offensive environment.

Materials and Methods

Bacterial strains and growth conditions

Bacillus subtilis strains 168 (*trpC2*) and QB4487 (*trpC2 degUΔBcl*I-*Eco*RI::*erm*) were used for the DegS/DegU experiments to generate data comparable with data published earlier. QB4487 was constructed from strain 168 [6], they were isogenic with the exception of a deletion in the *degU* gene in QB4487. For other experiments strain 1056 (wild type) has been used, because it is genetically closer to the Marburg strain.

Strain 1056 was cultivated at 37 ^{o}C in a water bath shaker in a modified synthetic minimal medium as described earlier [2] in order to apply nutrient limiting stress conditions. *B. subtilis* 168 and QB4487 were grown in LB liquid medium and on LB-agar plates, so that the results could be easily compared with other DegS/DegU experiments [7, 8]. Each experiment was repeated 7–11 times.

Stress conditions

Bacteria were stressed during the mid-exponential growth phase. Growing bacterial cultures were stressed (without collection and re-inoculation) in all cases with the exception of the nutrient limitation experiments. In all experiments two controls were taken directly before applying stress conditions.

In the case of acid shock, 25mM Tris/HCl and 25mM Na_3citrate/citric acid (pH 7.5) were used in the medium as a pH-buffer instead of 0.5M Tris/HCl buffer (pH 7.5) recommended by Stülke et al. [9]. In the case of phosphate-starvation, phosphate-limitation and carbon-limitation, the exponentially growing cultures were divided into two halves and the cells were collected separately. One half was resuspended in the original medium as a control and the other was in the limiting medium. For phosphate-starvation, the medium did not contain any KH_2PO_4. To evoke phosphate-limitation, the limiting medium contained 20 mM KH_2PO_4. In the case of carbon-limitation, the limiting medium contained 0.02 % (w/v) glucose. Oxygen-limitation was induced by incubating the cells at 37 ^oC in settled (non-stirred) culture flasks. For evoking salt stress, NaCl was used in a 6 % (w/v) final concentration**.** Equal amount of LB medium was added to the control samples.

In case of NaCl stress of the surface attached cells, the LB-agar of the stressed samples contained 6 % (w/v) of NaCl, whereas control bacteria were grown on LB-agar medium. Determination of the viable cell number was carried out in 3 duplicate samples; the standard deviation of the method was 8 %. Heat shock was induced by incubating the cells in a 45 ^oC water-bath shaker. For the induction of acid shock, the medium was set to pH=5.0 with 1 M HCl. To test the effect of a drop in nucleotide triphosphate concentrations, 2,4-dinitrophenol (DNP) was used in 300 μM final concentration, and mycophenolic acid (MPA) was applied in 0.045 μg/ml final concentration.

Determination of cell surface hydrophilicity

The determination of surface hydrophobicity was carried out following the rapid hexadecane-extraction method described earlier [10], with the following modifications: cells were killed immediately by pipetting them onto -20 ^oC ice containing 5 mM $MgCl_2$, 20 mM NaN_3 and 20 mM Tris/HCl at pH 7.5. Cells were collected, washed, and resuspended in physiological salt solution. The measured hydrophobicity was subtracted from 1 to obtain the hydrophilicity value. For the determination of the surface hydrophilicity during the various cell cycle phases under normal growth conditions the absolute hydrophilicity was calculated, whereas in cases of stresses the hydrophilicity of the stressed samples were compared to the untreated controls (relative

hydrophilicity). The results were only accepted if the controls were identical within an error of 4 %.

Determination of the intracellular ATP- and GTP-pool

Lysis of bacteria was brought about by adding 1/3 volume of 2 M ice-cold trichloroacetic acid (TCA) solution. The solution was prepared in pyrogen-free distilled water. Cells were collected by centrifugation for 3 minutes at 13000 g at +4 ^{o}C. 0.8 volume of the supernatant was discarded and the bacterial pellet was resuspended in the remaining 0.2 volume of the supernatant. Cells were then chilled at -80 ^{o}C for 20 minutes. Cell suspensions were thawed at room temperature and refrozen as above. Finally, the samples were thawed at room temperature and centrifuged at 13000 g for 10 minutes at +4 ^{o}C. Supernatants were either immediately subjected to HPLC-determination or stored at -20 ^{o}C until analysis.

The HPLC measurement was performed with a Gyncotek 480 pump. The separation of nucleotides was achieved on a Whatman Partisil PXS 10 / 25 SAX column at +25 ^{o}C, with an isocratic elution in 0.1 M potassium-phosphate buffer pH 6.0. The flow rate was 2.0 ml / minute. The volume of the loop was 100 µl. The detector was an UV TSK 6040 device set at 254 nm. Raw data were evaluated using Axxiom software. Relative nucleotide content was calculated as follows: the area of the nucleotide peak was integrated and compared to untreated control. These values were normalized to the OD_{550} of the cell suspension. The standard deviation of the method was 3 %.

Determination of the attachment to soil particles

Bacteria were cultivated in 25 ml of LB medium containing 1.5 g cellulose powder and 1.5 g washed river sand. The partition of the particle size of the river sand was: >0.005 mm 21.67 %, 0.05–0.01 mm 19.29 %, 0.01–0.02 mm 13.25 %, 0.02–0.05 mm 15.71 %, 0.05–0.1 mm 12.12 %, 0.1–0.15 mm 17.96 %. The sand was washed 3 times with 3 volumes of distilled water and dried at 60±5^{o}C for 5 days. Cell number was determined from the optical density measured at 550 nm, after filtering out the solids. Optical density of the sample taken after 30 minutes under stress was divided with the OD_{550} of controls, which was taken immediately before applying the stress (relative attachment value). The ratio between the relative attachment values of the samples and the same values of the non-stressed wild-type (strain 168) cells was expressed in percentage. Relative attachment value of 100% indicated that the same number of cells was found in the supernatant as in the controls. Values below 100% meant that cells attached stronger to the solid matrix then the control ones. The standard deviation of the method was 6 %.

Results

Description of the cell surface level response (CSLR)

Initially, the characteristic features of cell surface hydrophilicity of *B. subtilis*, as a measure of the CSLR, were determined during the various cell cycle phases under normal growth conditions in liquid medium. Fig. 1. shows the change of surface hydrophilicity in time. The surface was relatively hydrophilic in the lag-period, as well as in the early and late stationary phases, while cells became more hydrophobic during exponential growth. The lag-period and stationary phases are known as typical adaptation periods in which cells sense the changing environmental signals and try to respond to them. In these two phases, cells are particularly exposed to a number of stresses (changing or depleting carbon-, nitrogen-, energy-sources, pH, etc.). We have assumed that responses to some of these stress signals include alteration of the surface properties of the cells. It should be noted that no sporulation took place during the experiments because of the limited time period of investigations (only exponential and early-mid stationary phases were measured).

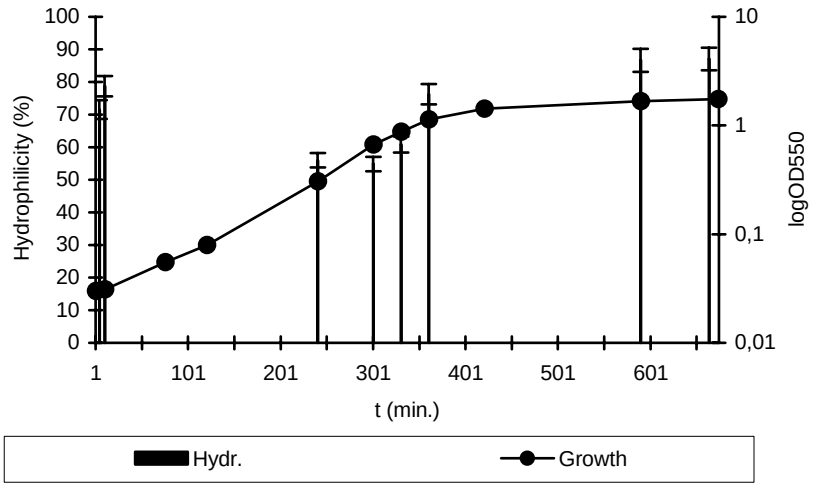

Fig. 1. The changes of surface hydrophilicity during the growth of bacteria.
The line demonstrates cell growth whereas the boxes show hydrophilicity of cell surfaces. For experimental procedures see: [10]. Error bars represent the standard deviation.

Fig. 2.A

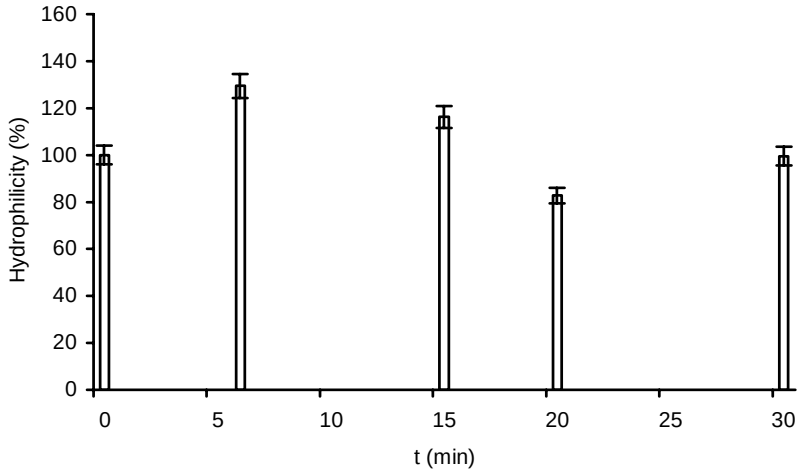
Hydrophilicity (%)
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
t (min)

Fig.2.B

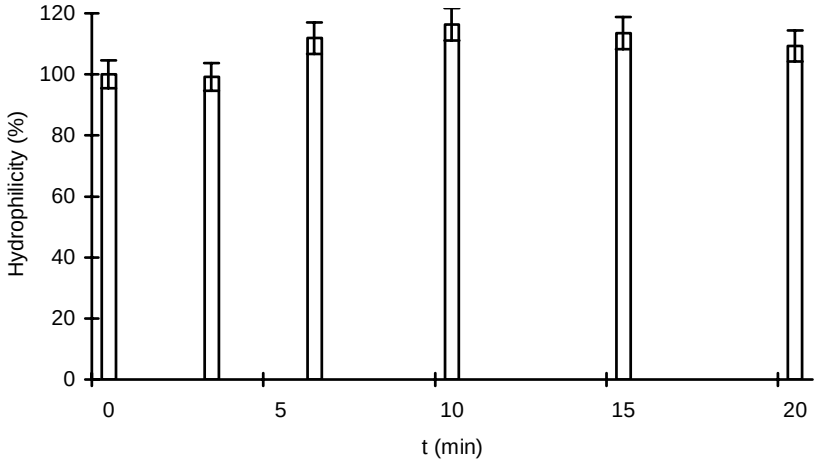
Hydrophilicity (%)
120
100
80
60
40
20
0
0
5
10
15
20
t (min)

Fig. 2.C

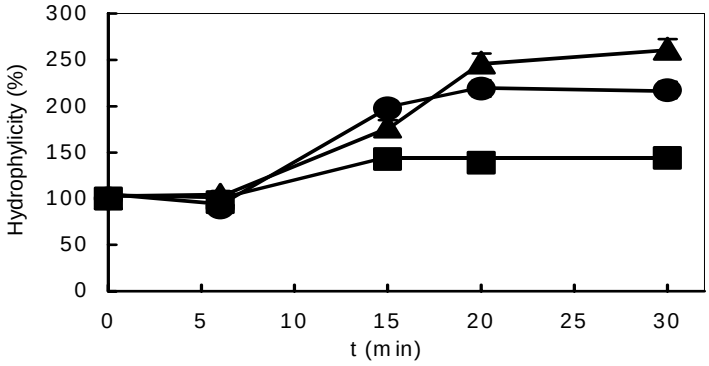

Fig. 2. The changes of the cell surface hydrophilicity after the impact of various stresses: (A) oxygen-limitation; (B) heat shock; (C) salt stress. The columns represent the relative surface hydrophilicity. For experimental procedures see: [10]. Error bars show the standard deviation. C: Symbols indicate the hydrophilicity of strain 1056 - ● -; 168 - ▲ - ; and QB4487 - ■ -, respectively.

B. subtilis immediately enters stationary phase if oxygen is limiting. Oxygen limitation, however, increased the surface hydrophilicity only moderately (Fig. 2.A).

In case of heat shock (Fig. 2.B), the CSLR was larger in amplitude. Following a pronounced increase in hydrophilicity during the first 10-15 minutes, a moderate decrease of surface hydrophilicity occurred. Thirty minutes after the provocation of cell response to heat shock, the cell surface hydrophilicity stabilized at the control level, indicating an acclimatization of the bacteria to the temperature shock.

A considerable increase of surface hydrophilicity was detected after exposure of cells to salt stress (Fig. 2.C). It commenced between the 6^{th} and the 15^{th} minutes, reached a maximum at the 20^{th} minute and remained at this level until, at least, 30 minutes after stressing the exponentially growing culture. It should be noted that the observed 219 % change in the hydrophilic-hydrophobic features of bacteria was the second highest ever detected as a result of bacterial stress response. (In the case of *Salmonella typhimurium* more than a 20-fold increase of hydrophobicity was measured in a response to mild acid (pH=6.0) stress [4]).

Lowering the pH of the medium to 5.0 did not markedly alter the surface hydrophilicity in *B. subtilis* (data not shown), similarly as observed in Gram negative bacteria [11].

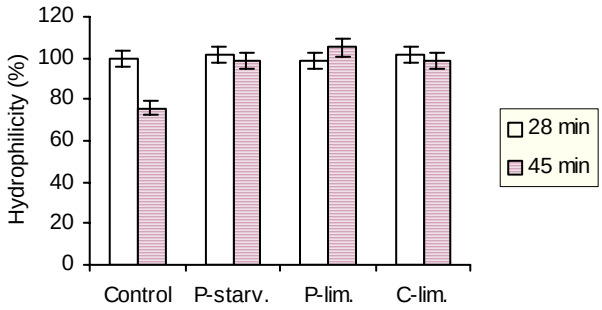

Fig. 3. Changes of the cell surface hydrophilicity under phosphate-limitation, carbon-limitation and phosphate-starvation, respectively.
Open bars demonstrate the surface hydrophilicity values of the samples in comparision with the controls, taken 28 minutes after resuspension of the bacteria in the media represented on the x axis. Filled columns show the hydrophilicity of the samples at 45 minutes. For experimental procedures see [10]. Stress conditions are described in the Materials and Methods section.

It is known that cells enter the stationary phase as soon as an essential component is depleted from the medium. This response is markedly different from a nutrient limitation induced specific response (e.g. phosphate or carbon limitation), in which the cells continue to grow but a signal of the suboptimal growth condition is sensed [12]. A manifestation of this phenomenon at CSLR level has been studied in detail.

In the phosphate-free medium, the bacteria showed a stationary phase-specific response. As evident from Fig. 3., complete phosphate-depletion of the medium caused an increase of surface hydrophilicity when compared to the untreated control in the 45 min observation period. The elevated hydrophilicity, measured during the first 30 minutes of the experiment, in both the controls and the stressed cells, is likely due to the mechanical stress of centrifugation and re-suspension. In case of phosphate- and carbon (energy)- limitation a nutrient-specific response was expected. However, an increase of cell surface hydrophilicity, very similar to phosphate starvation, was observed.

Because the CSLR induced by the stationary phase signals did not differ from that caused by either phosphate- or carbon (energy)- limitation specific responses, we concluded that, in all three cases, a general stress response was elicited from the bacteria, i.e. the observed CSLR was induced by general stress signals [12, 13, 14].

Fig. 4A

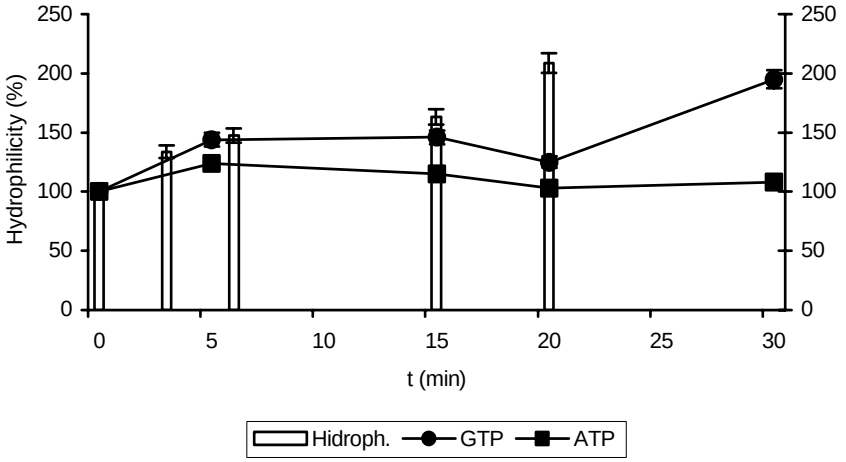

Fig. 4B

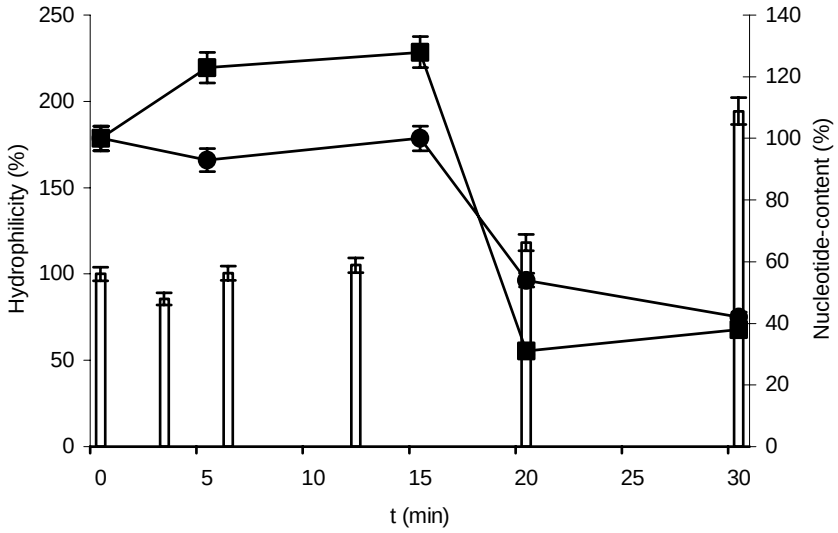

Fig. 4. Changes in the GTP- and ATP-pools and in the surface hydrophilicity of cells after treatment with (A) MPA or (B) 2,4-DNP. Circles indicate the GTP-pool of cells, whereas squares represent the changes in the ATP-pool of bacteria. Columns show the changes of the surface hydrophilicity in comparison with the untreated control. Error bars indicate the standard deviation. For experimental procedures see: [10], and the Materials and Methods section.

Intracellular signals of CSLR

It is important to establish the link between intracellular stress signal transduction mechanism(s) and CSLR as the bacteria enter into stationary phase or respond to various kinds of environmental stresses. Two potential parameters have been analysed: an energy limitation which, in most cases, induces the biosynthesis of general stress proteins [13, 15, 16], and a drop in GTP which leads to the onset of generalized physiological responses (e.g., sporulation) in *B. subtilis* [17, 18]. The intracellular responses have been followed by inhibitor studies. Although the drop of intracellular nucleotide levels is associated with the stress response, it is difficult to distinguish between primary and secondary effects. Therefore, the depletion of nucleotides can be either the cause or the result of a stress response. In any case nucleotide depletion can be viewed as part of the signal transduction pathway.

Mycophenolic acid (MPA) brings about a drop in GTP and ATP levels [19]. MPA acts as an inhibitor of inosite monophosphate (IMP)-dehydrogenase, the enzyme that is required for the synthesis of GMP. Therefore, in the presence of MPA, the GTP-pool in the cells decreases. It is also known that MPA strongly reduces the ATP-pool in cells [20]. This effect is most probably due to the phenomenon that adenylsuccinate-synthetase, an enzyme that is required for the synthesis of AMP, uses GTP as its energy-source. The surface hydrophilicity increased in a significant manner 20 minutes after the MPA-treatment (Fig. 4.A). Around the same time, a strong drop in ATP and GTP occurred. Thirty minutes after applying stress-conditions, the GTP-drop continued, however, the ATP-pool showed signs of recovery (Fig. 4.A). These results suggest that the drop of GTP and/or ATP may be an element of the signal-transducing process, which leads to the increased surface hydrophilicity following the MPA-treatment.

The effect of 2,4-dinitrophenol (2,4-DNP), which transports H^+-ions from the extracellular space into the cells, was also examined. Fig. 4.B demonstrates that the 2,4-dinitrophenol treatment caused a rapid, long-lasting and drastic increase of cell surface hydrophilicity. However, no intracellular ATP- or GTP-drop was detectable under our experimental conditions. On the contrary, a small but significant increase in the cellular ATP-pool and a drastically elevated GTP-level was found in response to the 2,4-DNP treatment. Therefore these results do not corroborate a direct relationship between the ATP- or GTP-drop in *B. subtilis* and its CSLR, in case of 2,4-DNP shock.

Role of the DegS/DegU two-component system in the CSLR

Wild type strain *B. subtilis* 168 and the *degU* deletion mutant strain QB4487 were subjected to NaCl stress in liquid medium and on solid surface. The results

observed in liquid medium indicated increased surface hydrophilicity following salt stress (Fig.2C). This was mainly due to the DegS/DegU-two component system in suspended culture as salt stress was accompanied by a significantly elevated surface hydrophilicity in the wild type *B. subtilis* compared to the *degU* deleted strain. It should be noted that a limited stress response was also detected in the mutant strain QB4487. The two wild type strains 1056 and 168 showed essentially the same behaviour regarding surface hydrophilicity. No differences were detected in the survival ability of strain 168 and QB4487 in response to salt stress (data not shown). Cells attached to agar plate containing 6 % (v/v) NaCl showed a distinct salt stress response (Fig. 5). Interestingly, the cell surface of the *degU* mutant QB4487 cells show essentially the same hydrophilicity on plate as wild-type strain 168, whereas the mutant cells were 7 times more resistant against salt stress than the wild-type strain.

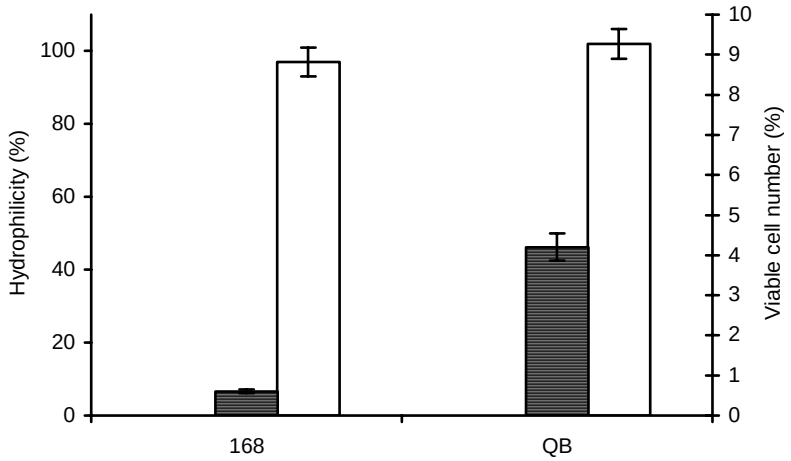

Fig. 5. Comparison of cell surface hydrophilicity and cell number properties of wild-type (168) and degU mutant (QB4487) attached cells under NaCl stress conditions.
Empty columns indicate cell surface hydrophilicity whereas hatched columns show cell number. Error bars indicate the standard deviation. For experimental procedures see the Material and Methods section.

Adhesion to solid particles in attachment-experiments indicated a defected response to salt stress conditions by the *degU* deleted cells, i.e. the stressed cells were strongly attached to the particles. No detectable differences were observed in the unstressed control samples, moreover the attachment properties of the wild type cells were the same in the presence and absence of the salt stress. It should be noted that the observed differences between the attachment properties of the strains QB4487 and 168

couldn't be explained by the different growth rate of the two strains. Unstressed QB4487 and 168 cells, similarly to NaCl-stressed QB4487 and 168 cells, grew identically in liquid medium (data not shown).

Discussion

The biosynthesis of a set of stress specific and general stress proteins is induced by environmental stimuli [14, 21, 22]. Defence mechanisms also include the synthesis of extracellular enzymes [9]. According to the results presented here, the complex modification of behaviour affects the cell adhesion features. In line with this conclusion, CSLR appeared as a general stress response and no variation in CSLR was found between C- and P-limitation. The pronounced effect induced by salt stress was remarkable. Salt stress was shown to activate the DegS-DegU two component system (for a review see [23]). The DegS-DegU system was probably also activated in strain 168 in a response to salt stress. This system has been implicated in the inhibition of the biosynthesis of the wall-associated high molecular mass WapA protein [6, 24]. Taken together, one can assume that the DegS-DegU system is participating in evoking a salt stress response through the inhibition of synthesis of WapA, which is likely to change the surface properties of the cells. DegS-DegU regulated processes are induced in the stationary phase [25, 26, 27], therefore their individual contribution to the increased surface hydrophilicity in the stationary phase (Fig. 1) cannot be distinguished.

According to the MPA inhibition experiments, a drop in intracellular GTP and/or ATP likely plays an important role in the signal transduction pathway. This trend was not corroborated by the 2,4-DNP inhibition studies. 2,4-DNP induced CSLR may be the result of a change in intracellular pH caused by 2,4-DNP, which invokes e.g. the synthesis of σ^B dependent gene products [28]. Therefore, we think that induction of CSLR should be occurred according to at least two pathways: an ATP and/or GTP-dependent and an -independent pathway, likely as it was described in case of the σ^B regulon [16].

An increase of cell surface hydrophilicity increases the probability that cells can disengage themselves from the soil particles [11]. In addition, cell motility can be increased through the induction of flagellin synthesis as part of the competence process regulated by the phosphorylation status of DegU [8, 29, 30]. The enhancement of motility of *B. subtilis* cells under growth-limiting conditions may be an important physiological function of CSLR to improve survival under unfavourable environmental conditions. The adhesion properties of the cells are important for numerous biotechnological applications where the binding of cells to carrier surface(s) is important for managing the active biomass within the fermentation space.

The DegS/DegU system does not appear to provide life saving function for the *B. subtilis* cells, since the deletion mutant cells were just as viable under stress condition as the wild type strain. Marked effects in cell surface hydrophilicity could be provoked by salt stress, although the cells behaved differently in suspended culture and on the surface of agar plates (Figs. 2.C. and 5). There is no clear explanation for the distinct surface properties, depending on the liquid *vs.* solid growth conditions, the changes should be linked to the DegS/DegU system.

Results of the attachment-experiments suggest that the DegS/DegU two-component system plays a crucial but complex role in responding to stress situation. The less hydrophilic mutant cells have certainly a lower chance to escape from salt stress when they were attached to a mixture of cellulose and sand particles. This strong attachment may be correlated with the hydrophilicity behaviour in solution, where the cells having a defected DegS/DegU system showed significantly lower surface hydrophilicity than the wild type controls, therefore they appeared to have restricted motility.

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