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ORIGINAL PAPER

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Bacillus subtilis functional genomics: genome-wide analysis of the DegS-DegU regulon by transcriptomics and proteomics

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Abstract The DegS-DegU two-component regulatory system of *Bacillus subtilis* controls various processes that characterize the transition from the exponential to the stationary growth phase, including the induction of extracellular degradative enzymes, expression of late competence genes and down-regulation of the σ^D regulon. The *degU32*(Hy) mutation stabilizes the phosphorylated form of DegU (DegU-P), resulting in overproduction of several extracellular degradative enzymes. In this study, the pleiotropic DegS-DegU regulon was characterized by combining proteomic and transcriptomic approaches. A comparative analysis of wild-type *B. subtilis* and the *degU32*(Hy) mutant grown in complex medium was performed during the exponential and in the stationary growth phase. Besides genes already known to be under the control of DegU-P, novel putative members of this regulon were identified. Although the *degU32*(Hy) mutant is assumed to contain high levels of phosphorylated DegU in the exponential as well as in the stationary growth phase, many genes known to be positively regulated by DegU-P did not show enhanced expression in the mutant strain during exponential growth. This is consistent with the fact that most genes belonging to the DegS-DegU regulon are subject to multiple regulation; this is also reflected in the strong stationary-phase induction of these genes in the mutant strain. As expected, during the exponential growth phase, the σ^D regulon was expressed at significantly lower levels in the *degU32*(Hy) mutant than in the wild type.

Keywords *Bacillus subtilis* · DegS-DegU regulon · Extracellular proteome · Transcriptome

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Introduction

Changes in the bacterial environment are frequently sensed by two-component regulatory systems that mediate adaptive responses to specific stimuli. Two-component systems consist of a sensor kinase and its cognate response regulator. The appropriate stimulus causes the autophosphorylating histidine protein kinase to transfer its phosphoryl group to the response regulator, which in most cases acts as a transcriptional activator in its phosphorylated state. In *Bacillus subtilis*, the DegS-DegU two-component system is involved in the complex network that mediates the regulation of transition state-specific processes. It contributes to the regulation of degradative enzyme synthesis, development of natural competence for DNA uptake and motility (Msadek et al. 1995). Phosphorylated DegU (DegU-P) mediates transcriptional activation of genes encoding degradative enzymes, whereas unphosphorylated DegU stimulates transcription of the gene *comK*, which encodes the competence transcription factor (Ogura and Tanaka 1996; Hamoen et al. 2000). In addition, genes controlled by the alternative sigma factor σ^D are negatively regulated by DegU-P (Msadek et al. 1993, Tokunaga et al. 1994; Rashid et al. 1996). The σ^D regulon encompasses genes involved in motility, chemotaxis and autolysin production (Marquez et al. 1990).

Two classes of mutations are known in both in *degS* and *degU*. One class of mutations leads to defects in the production of degradative enzymes, but the development of natural competence for DNA uptake is not affected. These mutations either result in synthesis of an

unphosphorylatable form of DegU or inactivate the DegS kinase function (Dahl et al. 1991). The second class of mutations leads to the overproduction of degradative exoenzymes (the Hy phenotype), correlated with the loss of natural competence for DNA uptake, lack of flagella synthesis, a filamentous morphology and higher sporulation efficiency in the presence of glucose (Kunst et al. 1974; Dahl et al. 1991, 1992). The *degS*(Hy) and *degU*(Hy) mutations provoke the accumulation of DegU-P either by increasing the phosphorylation rate and/or the stability of DegU-P, or by decreasing the rate of dephosphorylation of DegU-P by the DegS phosphatase activity (Tanaka et al. 1991; Dahl et al. 1992). The *degU32*(Hy) mutant utilized in the present study leads to a H→L substitution at position 12 in DegU (Henner et al. 1988a). This mutation increases the stability of the phosphorylated form of DegU sevenfold (Dahl et al. 1992).

Various extracellular enzymes involved in the degradation of proteins or carbohydrates were previously identified as members of the DegS-DegU regulon (Msadek et al. 1995). Recently, the proteomic approach was used to define the extracellular proteome of *B. subtilis* including the *degU32*(Hy) mutant (Antelmann et al. 2001). In this mutant, 13 degradative exoenzymes were overproduced. Furthermore, eight proteins involved in motility and cell-wall turnover synthesized in significantly decreased amounts; five of these latter proteins are encoded by σ^D-dependent genes (Antelmann et al. 2001). Besides the proteomic approach, the transcriptome analysis using DNA arrays represents a very useful tool for defining unknown regulons. Recently, the first global description of the DegS-DegU regulon was provided by Ogura and coworkers using DNA microarray technology (Ogura et al. 2001). In their experiments, a plasmid carrying the *degU* gene under the control of a P_{spac} promoter was introduced into a *degS* null mutant, and DegU overexpression was induced by the addition of IPTG. The results confirmed that several known DegU-P-activated genes, such as *aprE*, were induced upon overexpression of DegU. Consequently, the authors concluded that overexpression of DegU might mimic the function of the phosphorylated DegU. This approach allowed the identification of several putative new members of the DegS-DegU regulon.

In the present study, the gene expression patterns of the wild type and the *degU32*(Hy) mutant in complex medium were compared. The extracellular proteome and the corresponding transcriptome were analyzed in the exponential as well as in the stationary growth phase. This approach allowed the identification of new putative members of the DegS-DegU regulon.

Materials and methods

B. subtilis strains and growth conditions

The *B. subtilis* strains used in this study were *B. subtilis* 168 (*trpC2*) and *B. subtilis* MD300 (*trpC2*, *degU32*(Hy), Km^r). *B. subtilis* MD300 was constructed by transformation of *B. subtilis* QB4414 *degU146* (Dahl et al. 1991) with chromosomal DNA from *B. subtilis* QB136K1 (Msadek et al. 1991) and subsequent selection for kanamycin resistance (10 μg/ml). The *degU32*(Hy) phenotype was further verified by testing the transformants on skim milk plates. The strains were grown under vigorous agitation at 37°C in LB medium.

Transcriptome analysis by DNA macroarray hybridization

For the isolation of total RNA from *B. subtilis* strains, samples corresponding to 15 OD_{540} units were harvested during the exponential growth (OD_{540} = 0.4) and 1 h after the transition into stationary phase (OD_{540} = 3.5). Cell harvesting, preparation of RNA, synthesis of radioactively labelled cDNA and hybridization of *B. subtilis* macroarrays (Sigma-Genosys, The Woodlands, Tex., USA) were performed as described by Eymann et al. (2002). Each analysis was carried out twice, using two independently isolated RNA preparations and two different array batches. Exposed PhosphorImager screens were scanned with a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) at a resolution of 50 μm and a color depth of 16 bit.

For quantification of the hybridization signals and background subtraction, the ArrayVision software (Version 5.1, Imaging Research, St. Catherines, Ont., Canada) was used. Calculation of normalized intensity values of the individual spots was performed using the over-all-spot-normalization function of ArrayVision. To avoid extreme expression ratios for genes close to or below the detection limit, signal intensity values corresponding to a signal to noise ratio < 1.0 were scaled up to a value corresponding to a signal to noise ratio of 1.0. Further analyses were carried out using the GeneSpring 3.2.12 software (Silicon Genetics, Redwood City, Calif., USA). Genes exhibiting S/N ratios ≥3 under at least one growth condition were considered to be significantly expressed. All genes yielding signals below this significance threshold were excluded from further data analysis. Subsequently, the average of the normalized intensity values of the duplicate spots of each gene was used to calculate the expression level ratios. Induction or repression ratios ≥3 in both experiments were considered to be significant.

Final evaluation of the macroarray data included the consideration of putative operon structures derived from the genome sequence as well as previously known operons. Genes exhibiting significant expression ratios were analyzed for their transcriptional organization using the SubtiList database (http://genolist.pasteur.fr/SubtiList/). In cases where genes were members of polycistronic transcriptional units, further genes belonging to these operons were also included in the tables, even if their expression parameters did not meet the criteria for significance. Signal sequences and transmembrane segments in proteins were predicted using the PSORT algorithm (http://psort.nibb.ac.jp/).

Northern hybridization analysis

Northern analysis was carried out as described previously (Homuth et al. 1997). The digoxygenin-labelled specific RNA probes were synthesized by in vitro transcription using T7 RNA polymerase and specific PCR products as templates. Synthesis of the templates by PCR was performed using the following pairs of oligonucleotides: for the *aprE* probe, primers aprE5′ (5′-CTTTACGATGGCGTTCAGCA-3′) and aprE3′ (5′-CTAATACGACTCACTATAGGGAGAATTTGAGAAATGCCATAAGG-3′); for *hag*, hag5′ (5′-ATGAGAATTAACCACAATAT-3′) and hag3′ (5′-CTAATACGACTCACTATAGGGAGAAGTGTTTCCAGCTTGAACAA-3′); for *xylB*, xylB5′ (5′-TGAAGTATGTCATTG GAAT-3′) and xylB3′ (5′-CTAATACGACTCACTATAGGGAGACCAAAGAATTGCATTACGTA-3′); and for *wapA*, wapA5′ (5′-CTACAGAAGAAGAGAATGGA-3′) and wapA3′ (5′-CTAATACGACTCACTATAGGGAGATCAAAATAGCGTTCTCTGTC-3′).

Preparation of the extracellular protein fraction

B. subtilis cells were grown in 500 ml of LB medium and 250-ml samples were harvested during exponential growth (OD_{540} = 0.4) and 1 h after the transition into stationary phase. Cells were removed from the growth medium by centrifugation for 20 min at 5000×*g* and at 4°C. The proteins in the medium were then precipitated with ice-cold 10% (w/v) trichloroacetic acid (TCA), and collected by centrifugation (40,000×*g*, 45 min, 4°C). The resulting protein pellet was scraped from the wall of the centrifuge tube with a spatula, washed three times with 96% ethanol (v/v) and dried under vacuum.

Two-dimensional (2D) polyacrylamide gel electrophoresis

Dried protein pellets were dissolved in a solution containing 2 M thiourea and 8 M urea. Insoluble material was removed by centrifugation. The protein concentration of the resulting extracellular protein sample was determined as described by Bradford (1976), and a volume equivalent to 80 µg of protein was adjusted to 360 µl with the 2 M thiourea/8 M urea solution. Then, 40 µl of a 10-fold concentrated reswelling solution [2 M thiourea, 8 M urea, 10% Nonidet P-40, 200 mM DTT and 5% Pharmalyte (pH 3–10)] was added. This sample was used for the rehydration of IPG strips in the pH range 3–10 (Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing was performed using the Multiphor II unit (Amersham Biosciences, Freiburg, Germany) and SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Investigator 2-D electrophoresis system (Genomic Solutions, Chelmsford, Mass., USA) as described previously (Büttner et al. 2001). The resulting 2-D gels were fixed with 50% (v/v) methanol/7% (v/v) acetic acid, and stained with SYPRO Ruby protein gel stain (Molecular Probes, Eugene, Ore., USA). Fluorescence was detected using a Storm860 imager (Molecular Dynamics, Sunnyvale, Calif., USA). The 2-D gel image analysis and quantification were performed with the DECODON Delta 2-D software (http://www.decodon.com) which is based on dual-channel image analysis (Bernhardt et al. 1999). Using this software, the master image (represented by green spots) is warped onto the sample image (represented by red spots) after setting specific vector points. Consequently, the green protein spots are present in the master image only and the red protein spots are present in the sample image only. The yellow protein spots are present at similar amounts in both images. After background subtraction, normalization of the images was performed, equalizing the grey values of each image. For statistical reasons, samples from three independent experiments were resolved for each time point. The 2-D gels shown represent warped 2-D gels from one representative experiment.

Results

Quantitative analysis of the extracellular proteome of the *degU32*(Hy) strain

Recently, the extracellular proteome of wild-type *B. subtilis* during the exponential and the stationary growth phase in complex medium was defined, and found to comprise 82 extracellular proteins (Antelmann et al. 2001). In addition, comparison of the extracellular proteome of the wild type to that of the *degU32*(Hy) strain revealed up-regulation of 13 degradative enzymes and down-regulation of eight proteins involved in motility and cell-wall turnover in stationary-phase cells of the *degU32*(Hy) mutant (Antelmann et al. 2001). In agreement with these findings, earlier studies had shown that the genes encoding the proteases AprE and NprE are positively regulated by DegU-P (Msadek et al. 1995). In the present work, one further protein (BglC) was identified which appears to be positively regulated by DegU-P. To complete this purely descriptive approach, the ratios of the positively regulated extracellular enzymes (relative to the wild type) were calculated using the quantitation tool in the DECODON Delta 2D software (Figs. 1A and 2A). Whereas the relative amounts of AmyE, Ggt, Vpr and YnfF were only 2–3 times higher in the mutant, ratios between 10 and 33 were obtained for AprE, BglC, BglS, Bpr, Mpr, NprE, PelB, YfkN, YwaD and YurI. As an internal control, the extracellular proteins YfnI and YxaL were quantified. These proteins are obviously not regulated by DegU-P, as revealed by the proteome analyses. The ratios obtained for them were 1.08 and 0.88, respectively. The levels of eight extracellular proteins were found to be strongly reduced in the *degU32*(Hy) mutant (Fig. 1A). Of the down-regulated proteins, the autolysins LytD and YwtD, the flagellin Hag, as well as the flagellar hook-associated proteins FlgK and FliD are encoded by σ^D-dependent genes.

The extracellular proteomes of the *degU32*(Hy) mutant in the exponential growth phase and in the stationary growth phase were compared in order to analyze the secretion pattern of proteins encoded by genes controlled by DegU-P (Fig. 1B). It turned out that some of the regulated degradative enzymes were only weakly synthesized in the exponential growth phase and strongly induced in stationary phase (Figs. 1B and 2B). For example, the aminopeptidase YwaD, the glucanase BglC and the putative nucleotidases YfkN and YurI were absent from the extracellular proteome of the *degU32*(Hy) mutant during exponential growth. These proteins were induced at least 10-fold in the stationary phase. Other degradative enzymes including AprE, AmyE, BglS, PelB, Mpr, NprE and Vpr, were present in 2- to 4-fold higher amounts in the stationary phase. Of the two control proteins, YfnI was not synthesized in the exponential growth phase and therefore, only YxaL could be quantified. The YxaL protein exhibited a ratio of 0.87.

The transcriptome of the *degU32*(Hy) strain in the exponential and the stationary growth phase

To obtain a more complete picture of the DegS-DegU regulon, a macroarray analysis was carried out in addition to the characterization of the extracellular proteome. Putative DegU-P-regulated genes were identified by significantly altered expression levels in the *degU32*(Hy) mutant compared to the wild type, either in the exponential or in the stationary growth phase. Most probably, in the *degU32*(Hy) mutant higher levels of the phosphorylated form of DegU are present during both exponential and stationary growth phases. In contrast, the wild type is thought to contain mainly unphosphorylated DegU during exponential growth, whereas the transition into the stationary phase most probably

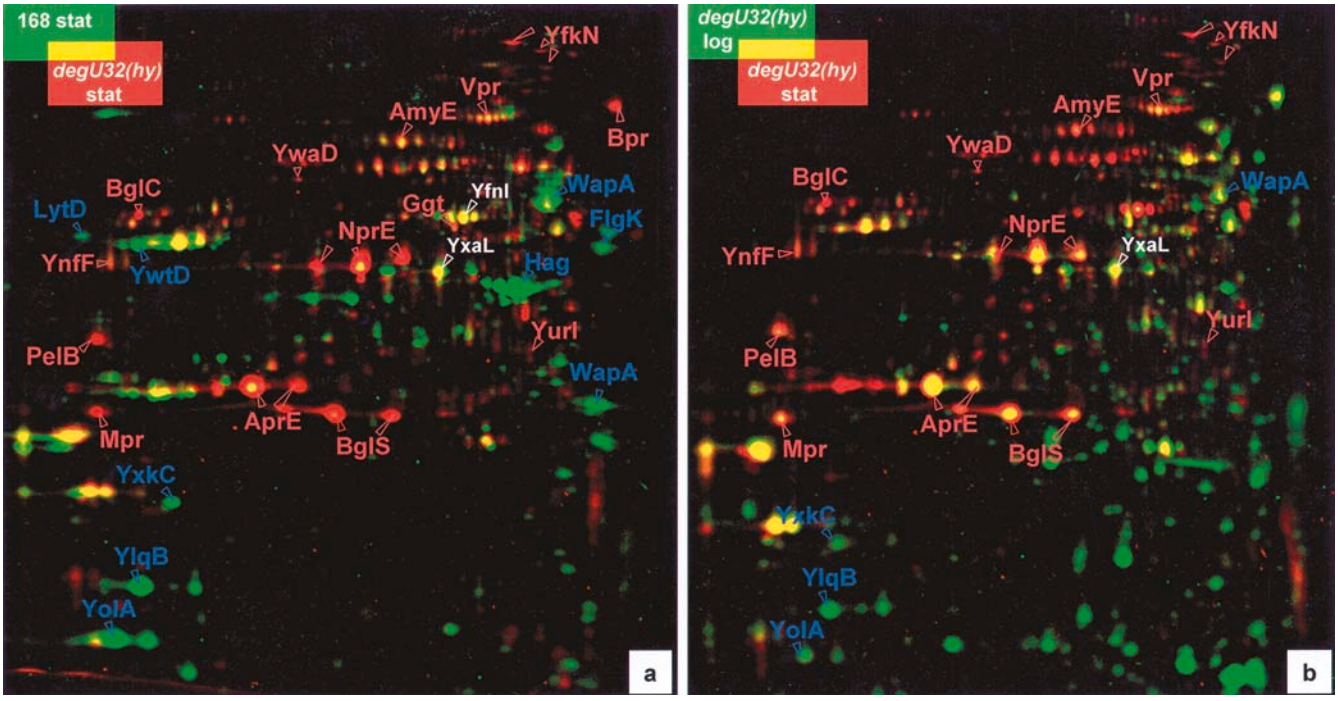

Fig. 1A The stationary-phase extracellular proteome of the *B. subtilis degU32*(Hy) mutant (*red image*) in comparison to the wild-type (168) (*green image*). Extracellular proteins were separated as described in Materials and methods. Protein spots that decreased in intensity in the *degU32*(Hy) mutant are labelled in *blue* and spots that increased are labelled in *red*. Spots labelled in *white* represent proteins that are not regulated by DegU-P and served as an internal control. **B** The extracellular proteome of the *B. subtilis degU32*(Hy) mutant during the exponential growth (*green image*) in comparison to the stationary phase (*red image*). Protein spots that decreased in intensity in the stationary phase are labelled in *blue* and spots that increased are labelled in *red*. The YxaL protein (labelled in white) was quantified as an internal control

induces DegU phosphorylation by its specific ATP-dependent kinase DegS.

The comparison of the wild-type transcriptome to that of the *degU32*(Hy) mutant in the exponential growth phase was expected to allow the identification of genes that are positively regulated by DegU-P. However, those DegU-P regulated genes which are strongly repressed by other regulatory proteins during exponential growth or which require additional activation for full expression in the stationary phase should be missing from the list of DegU-P-regulated proteins. These genes should show an increased expression level in the *degU32*(Hy) mutant compared to the wild type only in the stationary phase. Furthermore, DegU-P-dependent genes which are repressed during exponential growth were expected to show a stationary phase induction in both strains. In total, the transcripts of 32 genes were detected in at least 3-fold higher amounts in the *degU32*(Hy) mutant, yielding induction ratios ≥3 in both parallel experiments (Table 1). Of these genes, 18 were significantly more strongly expressed in the *degU32*(Hy) mutant during exponential growth and 14 in the stationary growth phase.

Among the 18 genes exhibiting significantly higher mRNA levels in the *degU32*(Hy) mutant during the exponential growth phase (Table 1; hy/wt log), only *sacB* (encoding levansucrase) was previously reported to be regulated by DegU-P (Crutz and Steinmetz 1992). The *degS-degU* operon encodes the DegS sensor kinase and the DegU response regulator (Henner et al. 1988b, Msadek et al. 1993). The significant up-regulation of the *degU* gene itself suggested the presence of an internal promoter immediately upstream of *degU*. Indeed, previous work has provided evidence for the existence of a promoter at the 3′ end of the *degS* coding region (Msadek et al. 1990). Our results demonstrate positive regulation of this promoter by DegU-P, indicating a positive feedback mechanism. Altogether, 16 y-genes encoding products of still unknown function were expressed at significantly higher levels in the *degU32*(Hy) mutant. According to the SubtiList database, most of the derived gene products did not show significant similarities to proteins of known functions. Obviously, the corresponding transcriptional units are not, or only weakly, repressed by other regulatory proteins in the exponential growth phase. In the *degU32*(Hy) mutant, the majority of the genes exhibiting higher levels of expression during exponential growth were down-regulated in the stationary phase. This expression pattern might be attributable to specific regulatory mechanisms triggered by components of the complex LB medium.

In the stationary growth phase, 14 genes exhibited significantly higher levels of mRNA in the *degU32*(Hy) mutant compared to the wild type (Table 1; hy/wt stat). Of these genes, *amyE* (encoding alpha-amylase), *aprE* (encoding an extracellular serine protease), *ispA* (encoding an intracellular serine protease) and *nprE*

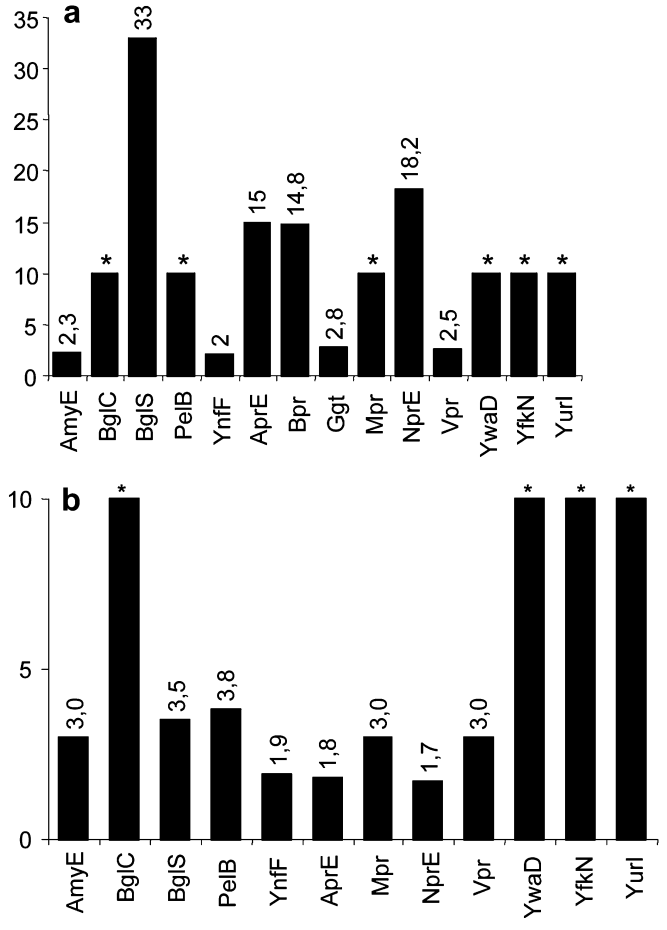

Fig. 2 Induction ratios of DegU-P regulated extracellular proteins. The values in the diagrams represent induction ratios which were obtained either by comparing the *degU32*(Hy) mutant and the wild type in the stationary phase (**A**) or stationary phase and exponential growth phase in the *degU32*(Hy) mutant (**B**). Relative induction ratios in the diagrams were calculated based on the quantification of the extracellular proteomes shown in Figs. 1A and B, respectively. The *asterisks* indicate the absence of detectable protein spots in the wild-type strain (**A**) and in the exponential growth phase (**B**), respectively. In these cases, infinite induction ratios were obtained using the DECODON Delta 2-D software, and induction ratios were arbitrarily set to 10

(encoding an extracellular metalloprotease) were previously known to be regulated in a DegU-P-dependent manner (Ruppen et al. 1988; Msadek et al. 1995; Antelmann et al. 2001). Other up-regulated genes were *csn* (encoding chitosanase), *spoVR* (encoding a sporulation protein) and 8 y-genes. Although in the *degU32*(Hy) mutant the phosphorylated form of DegU is present at high levels during the exponential and stationary growth phases, this group of DegU-P-regulated genes was significantly induced in stationary phase in the *degU32*(Hy) mutant. During exponential growth, negative regulatory effects seem to prevent DegU-P mediated transcriptional activation. In agreement with this observation, most genes that are positively regulated by DegU-P are known to be repressed by other regulatory proteins during exponential growth. Transcription of *amyE* is repressed by CcpA in the presence of glucose (Henkin et al. 1991) and *aprE* is transcriptionally repressed by the transient phase regulators ScoC (Henner et al. 1988a; Kallio et al. 1991), AbrB (Ferrari et al. 1988; Olmos et al. 1996) and SinR (Olmos et al. 1997). Negative regulation by ScoC has also been described for *nprE* (Kallio et al. 1991), and expression of *bglS* was found to be regulated in response to the level of GTP in the cell, most probably also mediated by AbrB (Stülke et al. 1993).

Genes that are negatively regulated by DegU-P

Table 2 presents the repression ratios of negatively DegU-P regulated genes in the *degU32*(Hy) mutant compared to the wild type. During the exponential growth phase, hyperphosphorylation of DegU in the *degU32*(Hy) mutant results in down-regulation of the complete σ^D regulon. In the case of the *fla/che* operon, which encompasses more than 30 genes distributed over more than 24 kb, almost all of the genes expressed significantly lower amounts of mRNA levels in the mutant. This operon is transcribed from two promoters upstream of *flgB*: P_A is recognized by the σ^A RNAP and P_{D-3} by the σ^D RNAP (Marquez et al. 1990; Marquez-Magana and Chamberlin 1994; Estacio et al. 1998; Yang et al. 1999; West et al. 2000). The following transcriptional units, which were known to be at least partially σ^D dependent, shared this specific expression pattern: the *flgM-yvyG-flgKL* operon (Mirel et al. 1994), the *fliDST* operon (Chen and Helmann 1994), the *lytABC* operon (Lazarevic et al. 1992; Kuroda and Sekiguchi 1993), the *motAB* operon (Mirel et al. 1992) and the monocistronic genes *lytF* (Margot et al. 1999), *mcpC* (Müller et al. 1997) and *hag* (Mirel and Chamberlin 1989). Furthermore, the monocistronic genes *mcpA*, *mcpB*, *yvzB*, *ywtD*, and *yxkC*, as well as the bicistronic operons *yfmTS*, *yolAB* and *flhOP*, were expressed at significantly lower levels in the *degU32*(Hy) mutant. The products encoded by *ywtD* (a murein hydrolase homologue), *yxkC* and *yolA* were recently found to be absent from the extracellular proteome of a *sigD* mutant (Antelmann et al. 2002). The *yvzB* gene, whose product shows strong similarity to flagellin, might also be classified as a member of the σ^D regulon. The *flhOP* operon encoding flagellar proteins, and the *mcpA* and *mcpB* genes encoding methyl-accepting chemotaxis proteins (Hanlon and Ordal 1994), might represent further members of this regulon. On the basis of its expression pattern and protein sequence homologies, the *yfmTS* operon (whose products are similar to benzaldehyde dehydrogenase and a methyl-accepting chemotaxis protein) possibly belongs to the σ^D regulon too.

Besides the members of the σ^D regulon, several other genes were expressed at reduced levels in the *degU32* (Hy) mutant, among them the *srfAAABACAD* operon which encodes the surfactin synthetase and the competence regulatory factor ComS (D'Souza et al. 1994). Reduced expression of this operon in a *degU32*(Hy)

Table 1 Genes showing significantly elevated expression in *B. subtilis degU32*(Hy) compared to wild-type *B. subtilis* 168, either in the exponential or in the stationary growth phase as revealed by transcriptome and proteome analyses

Gene[a]	Ratio[b]				Transcriptional organization[c]	Product function[d]
	hy/wt (log)	hy/wt (stat)	stat/log (wt)	stat/log (hy)		
amyE	0.9, –	**3.0, 3.5**	2.7, –	**9.3, 4.8**	***amyE***	Alpha-amylase; SS
aprE	0.6, 2.1	**29.4, 29.1**	**3.2, 11.3**	**145.6, 154.3**	***aprE***	Serine alkaline protease (subtilisin E); SS
bglC	0.3, 0.3	0.7, 1.1	0.5, 0.3	1.2, 1.2	***bglC***	Endo-1,4-beta-glucanase; SS
bglS	–, –	2.2, 3.4	–, –	**3.0, 7.7**	***bglS***	Endo-beta-1,3–1,4 glucanase; SS
bpr	–, –	3.0, –	–, –	3.8, –	***bpr***	Bacillopeptidase F; SS
csn	0.8, 1.1	**5.9, 5.9**	**6.8, 11.3**	**51.0, 63.2**	***csn***	Chitosanase; SS
degS	1.1, 1.0	0.8, 1.0	1.0, 1.5	0.7, 1.6	*degS*-***degU***	Two-component histidine kinase
degU	**6.8, 6.5**	1.7, 1.4	**3.0, 4.2**	0.7, 0.9	*degS*-***degU***	Two-component response regulator
ggt	–, –	1.2, 3.4	3.5, –	**5.3, 12.9**	***ggt***	Gamma-glutamyltranspeptidase; SS
ispA	2.2, 3.0	**10.8, 8.9**	**3.3, 3.8**	**15.9, 10.8**	***ispA***	Intracellular serine protease
mpr	–, –	1.7, 1.3	–, –	**9.1, 3.9**	***mpr***	Extracellular metalloprotease; SS
nprE	2.4, 7.2	**11.7, 12.5**	2.2, 7.3	**10.7, 12.6**	***nprE***	Extracellular neutral metalloprotease; SS
pelB	0.6, –	0.9, 1.0	1.2, –	1.8, 1.7	***pelB***	Pectate lyase; SS
sacB	**3.9, 4.7**	–, –	–, –	0.2, 0.5	***sacB***	Levansucrase; SS
spoVR	1.1,1.5	**5.2, 3.1**	1.2, 2.4	**5.5, 4.8**	***spoVR***	Involved in spore cortex synthesis
vpr	0.6, 1.0	0.7, 0.6	**5.1, 14.7**	**6.1, 9.0**	***vpr***	Minor extracellular serine protease; SS
ycdA	**8.6, 14.0**	0.7, –	2.1, 2.2	0.2, 0.04	***ycdA***	Similar to membrane lipoprotein
yddT	1.1, 1.3	**10.5, 4.2**	1.4, 3.2	**13.8, 10.3**	***yddT***	Unknown function; SS
yfjA	**4.0, 10.0**	1.2, 0.9	–, –	0.7, 0.5	***yfjA-yfjB-yfjC-yfjD***	Unknown function
yfjB	**10.7, 14.4**	–, –	–, –	0.1, 0.3	***yfjA-yfjB-yfjC-yfjD***	Unknown function; 2 TMS
yfjC	**6.2, 14.0**	0.6, 1.9	–, –	0.2, 0.5	***yfjA-yfjB-yfjC-yfjD***	Unknown function
yfjD	**4.6, 5.5**	–, 1.1	–, –	0.3, 1.3	***yfjA-yfjB-yfjC-yfjD***	Unknown function; 2 TMS
yfkN	1.0, 1.5	0.5, 1.9	**6.1, 6.7**	**3.4, 8.5**	***yfkN***	Similar to 2′,3′-cyclic-nucleotide phosphodiesterase; SS
yitN	–, –	–, –	–, –	–, –	*yitN*-***yitM***	Unknown function; 3 TMS
yitM	–, 1.6	**3.5, 9.0**	2.0, 6.4	**8.8, 36.7**	*yitN*-***yitM***	Unknown function; SS; 1 TMS
yjhA	**9.5, 17.8**	–, –	0.7, 1.0	0.03, 0.04	***yjhA-yjhB***	Putative lipoprotein;
yjhB	**3.0, 3.1**	0.4, 0.5	2.1, 3.5	0.3, 0.5	***yjhA-yjhB***	Similar to mutator MutT protein
ynfF	–, –	1.1, 1.4	–, –	**4.2, 6.6**	***ynfF***	Similar to endo-xylanase; SS
yoaJ	–, 2.6	**6.6, 8.6**	–, 2.8	**12.3, 9.3**	***yoaJ***	Similar to extracellular endoglucanase; SS
yomL	–, –	**6.2, 4.0**	–, –	**18.8, 11.9**	***yomL***	Unknown function; SS
yqxI	**6.7, 10.3**	2.1, 5.5	**7.2, 25.4**	**3.2, 13.8**	***yqxI-yqxJ***	Unknown function; SS
yqxJ	–, –	2.1, 3.0	–, –	**3.1, 9.1**	***yqxI-yqxJ***	Unknown function
yraI	–, –	**3.4, 16.9**	–, –	**8.5, 54.1**	***yraI-yraJ***	Unknown function; 1 TMS
yraJ	–, –	**4.7, 10.0**	–, –	**7.8, 24.6**	***yraI-yraJ***	Unknown function; SS
ytvB	–, –	**8.1, 3.0**	–, –	**16.4, 9.9**	***ytvB***	Unknown function; SS; 2 TMS
yuiI	**3.1, 3.5**	–, –	0.3, 0.05	0.03, 0.03	***yuiI***	Unknown function; 1 TMS
yukE	**10.7, 5.7**	0.4, 0.6	**3.3, 3.8**	0.1, 0.4	***yukE***-*yukD*-***yukC***-*yukB*-***yukA***-*yueB*-*yueC*-*yueD*	Unknown function
yukD	2.1, 1.8	0.6, 0.9	–, 1.9	0.5, 1.0	***yukE***-*yukD*-***yukC***-*yukB*-***yukA***-*yueB*-*yueC*-*yueD*	Unknown function
yukC	**4.7, 4.0**	–, –	0.6, 0.8	0.04, 0.1	***yukE***-*yukD*-***yukC***-*yukB*-***yukA***-*yueB*-*yueC*-*yueD*	Unknown function; 1 TMS
yukB	1.7, 2.0	0.7, 0.9	–, 2.9	0.9, 1.3	***yukE***-*yukD*-***yukC***-*yukB*-***yukA***-*yueB*-*yueC*-*yueD*	Unknown function; SS; 1 TMS
yukA	**6.7, 4.8**	0.4, 0.7	1.6, 1.7	0.1, 0.2	***yukE***-*yukD*-***yukC***-*yukB*-***yukA***-*yueB*-*yueC*-*yueD*	Unknown function; ATP/GTP-binding site motif
yueB	1.5, –	–, –	1.2, –	0.4, –	***yukE***-*yukD*-***yukC***-*yukB*-***yukA***-*yueB*-*yueC*-*yueD*	Unknown function; SS; 6 TMS
yueC	2.3, 0.5	–, –	1.1, 0.6	0.3, –	***yukE***-*yukD*-***yukC***-*yukB*-***yukA***-*yueB*-*yueC*-*yueD*	Unknown function; SS; 1 TMS
yueD	2.1, 2.2	–, –	–, –	0.4, 0.5	***yukE***-*yukD*-***yukC***-*yukB*-***yukA***-*yueB*-*yueC*-*yueD*	Similar to sepiapterin reductase
yurI	0.9, 1.0	2.1, 4.2	3.0, 2.7	**6.6, 10.9**	***yurI***	Similar to ribonuclease; SS
yvpA	–, 1.5	3.3, 3.7	–, –	**5.4, 4.6**	***yvpA***	Similar to pectate lyase; SS
ywaD	–, –	2.4, 3.0	**9.5, 9.7**	**14.0, 18.3**	***ywaD***	Similar to aminopeptidase; SS
ywqH	2.6, 3.5	2.6, 0.8	**3.7, 3.7**	3.6, 0.9	*ywqH*-***ywqI-ywqJ***-*ywqK*-*ywqL*	Unknown function
ywqI	**3.4, 4.1**	2.0, 0.5	–, –	2.6, 0.7	*ywqH*-***ywqI-ywqJ***-*ywqK*-*ywqL*	Unknown function
ywqJ	**3.8, 4.0**	3.0, 0.6	**6.2, 10.7**	4.9, 1.6	*ywqH*-***ywqI-ywqJ***-*ywqK*-*ywqL*	Unknown function
ywqK	–, –	2.0, –	–, –	2.6, 1.1	*ywqH*-***ywqI-ywqJ***-*ywqK*-*ywqL*	Unknown function
ywqL	1.3, 2.2	1.1, 0.7	**3.0, 4.2**	2.3, 1.3	*ywqH*-***ywqI-ywqJ***-*ywqK*-*ywqL*	Similar to putative endonuclease

Table 1 (Contd.)

Gene[a]	Ratio[b]				Transcriptional organization[c]	Product function[d]
	hy/wt (log)	hy/wt (stat)	stat/log (wt)	stat/log (hy)		
ywsC	2.6, 3.4	–, –	–, –	0.2, 0.3	*ywsC-ywtA-**ywtB-ywtC***	Similar to *B. anthracis* CapB; SS; 2 TMS
ywtA	1.4, –	–, –	–, –	0.6, 1.2	*ywsC-ywtA-**ywtB-ywtC***	Similar to *B. anthracis* CapC; SS; 5 TMS
ywtB	**7.6, 15.7**	2.1, –	–, –	0.3, 0.2	*ywsC-ywtA-**ywtB-ywtC***	Similar to *B. anthracis* CapA; 1 TMS
ywtC	**4.0, 4.6**	–, –	–, –	0.4, 0.3	*ywsC-ywtA-**ywtB-ywtC***	Unknown function; SS

[a]Gene names highlighted in *bold* indicate higher expression in *B. subtilis degU32*(Hy) as revealed by proteome analysis
[b]The calculated expression level ratios are shown for the two independent macroarray experiments performed (– indicates that the signals were below the significance threshold). log, exponential phase of growth; stat, stationary growth phase. Ratios shown in *bold* indicate significant up-regulation, defined as a ≥3-fold change in mRNA levels in both macroarray experiments
[c]Gene names depicted in *bold* indicate significant up-regulation of these loci in the exponential or the stationary growth phase in *B. subtilis degU32*(Hy). Genes identified by Ogura et al. (2001) as putative members of the DegU regulon are *underlined*
[d]SS and TMS indicate the presence of signal sequences and transmembrane segments, respectively, as predicted by *in silico* analysis of the encoded proteins

background has already been described (Hahn and Dubnau 1991). The bicistronic operon *wapA-yxxG* also turned out to be down-regulated in the mutant strain. The WapA protein encoded by this operon represents a cell wall-associated protein precursor. Its negative regulation by DegU-P under conditions of salt stress conditions was reported previously (Dartois et al. 1998). Other genes identified as being directly or indirectly down-regulated by the phosphorylated form of DegU in the exponential growth phase encode products involved in various cellular functions including amino acid transport (*aapA*), protein modification (*amhX*), lipoic acid synthesis (*lipA*), carbohydrate metabolism (*pckA*, *xylAB*), teichoic acid biosynthesis (*tagC*), sporulation (*sspB*) and detoxification (*mmr*). In addition, a total of 41 ORFs with unknown functions were down-regulated in the *degU32*(Hy) mutant. Of the derived gene products, 19 show similarity to known proteins, but no common physiological function could be discerned for these genes.

The majority of the genes belonging to the σ^D regulon exhibited significant differences in mRNA levels between the *degU32*(Hy) mutant and wild type only during the exponential growth phase. In the stationary growth phase, the induced DegU phosphorylation provoked the expected strong repression of the σ^D regulon in the wild type, resulting in clearly lower expression ratios for these genes or in signals below the significance threshold in both strains. Only the σ^D-dependent genes *fliI*, *hag*, *lytF* and *mcpC* showed significant repression in the *degU32*(Hy) mutant relative to the wild type. Further genes that are down-regulated in the mutant strain are involved in the degradation of carbohydrates (*ynaJ-xynB*, *xylAB*), surfactin biosynthesis and regulation of competence (*srfAAABACAD*). Interestingly, the *srfAAABACAD* operon was strongly induced during stationary phase in the wild type and in the mutant strain, but in the latter the basal expression level was significantly reduced.

Verification of global expression analyses by Northern hybridization

To verify the results obtained in the proteome and transcriptome studies, four selected genes were analyzed in detail by Northern hybridization (Fig. 3). In the case of *aprE*, the global expression analyses revealed nearly 30-fold more mRNA and 15-fold more protein in the *degU32*(Hy) mutant compared to the wild type in the stationary phase (Table 1, Fig. 2A). In agreement with this, the Northern blot showed higher amounts of the *aprE*-specific 1.2-kb transcript in the mutant strain primarily in the stationary growth phase. Furthermore, the Northern analysis confirmed the very strong induction of *aprE* in the mutant strain in stationary phase revealed by the array analysis.

The analysis of the extracellular proteome revealed a significant down-regulation of *wapA* and the σ^D-dependent *hag* gene in the *degU32*(Hy) mutant (Fig. 1A). According to the transcriptome analysis (Table 2), the *hag* gene, which codes for flagellin expressed around 30-fold less mRNA in the exponential phase and around 6-fold less mRNA in the stationary phase in the mutant strain compared to the wild type. The Northern blot experiment verified these results. In both growth phases, much more *hag*-specific 1.0-kb mRNA was detected in the wild type than in the mutant strain, whereby the wild-type transcript was down-regulated in the stationary phase. Consistently with the array analyis, the Northern data for *hag* showed that the phosphorylation of DegU induced in stationary phase caused repression of the σ^D regulon in the wild type. The remarkably weak down-regulation of the *hag* mRNA in stationary phase might be reflection of the extraordinarily long half-life of this transcript (S. Hennig and G. Homuth, unpublished results).

The *wapA* and *yxxG* genes produced around 10-fold less mRNA in the *degU32*(Hy) mutant during the exponential growth phase, whereas in the stationary phase very low levels of these mRNAs were present in both

Table 2 Genes showing significantly decreased expression in *B. subtilis degU32*(Hy) compared to *B. subtilis* 168 wild type either in the exponential or in the stationary growth phase as obtained by transcriptome and proteome analyses

Gene[a]	Ratio[b]				Transcriptional organization[c]	Product function[d]
	wt/hy (log)	wt/hy (stat)	log/stat (wt)	log/stat (hy)		
aapA	**3.3, 6.0**	2.0, –	2.5, 3.2	–, –	***aapA***	Amino acid permease; 11 TMS
amhX	**3.7, 6.7**	1.5, 1.2	1.4, 2.7	0.6, 0.5	***amhX***	Amidohydrolase
flgB	3.2, 2.3	–, –	1.5, 1.6	–, –	flgBC-fliEFGHIJ-***ylxF-fliK-ylxG***-flgE-***fliLMY-cheY-fliZ***PQR-*flhBAF-ylxH-cheBA****WCD-sigD-ylxL***	Flagellar basal-body rod protein
flgC	**6.4, 7.5**	4.8, 2.2	1.3, 1.0	–, –	*fliBC–ylxL*	Flagellar basal-body rod protein
fliE	**3.6, 3.5**	1.4, 1.7	1.2, 1.0	0.5, 0.5	*fliBC–ylxL*	Flagellar hook-basal body protein
fliF	**12.2, 15.5**	–, 7.7	2.5, 1.4	–, –	*fliBC–ylxL*	Flagellar basal-body M-ring protein; 2 TMS
fliG	**13.7, 14.6**	–, –	3.0, 1.8	–, –	*fliBC–ylxL*	Flagellar motor switch protein; SS; 1 TMS
fliH	**7.2, 8.8**	–, –	2.6, 2.3	–, –	*fliBC–ylxL*	Flagellar assembly protein
fliI	**7.2, 8.2**	**4.4, 4.0**	1.3, 0.5	0.8, 0.3	*fliBC–ylxL*	Flagellar-specific ATP synthase
fliJ	**3.8, 3.3**	–, –	2.3, 1.1	–, –	*fliBC–ylxL*	Required for formation of basal body
ylxF	**3.8, 4.4**	–, –	2.8, 2.2	–, –	*fliBC–ylxL*	Unknown function; SS
fliK	–, –	–, –	–, –	–, –	*fliBC–ylxL*	Flagellar hook-length control
ylxG	**10.2, 11.1**	–, –	2.8, 2.1	–, –	*fliBC–ylxL*	Similar to flagellar hook assembly protein
flgE	**13.4, 10.1**	2.4, –	2.4, 1.7	–, –	*fliBC–ylxL*	Flagellar hook protein
fliL	**12.2, 9.3**	–, –	**4.4, 3.1**	–, –	*fliBC–ylxL*	Required for flagellar formation; SS
fliM	**6.5, 3.7**	–, –	2.3, 1.2	–, –	*fliBC–ylxL*	Flagellar motor switch protein; 1 TMS
fliY	**10.9, 13.4**	3.3, 2.7	**5.6, 4.9**	1.7, 1.0	*fliBC–ylxL*	Flagellar motor switch protein
cheY	**4.5, 4.4**	–, –	2.6, 1.5	–, –	*fliBC–ylxL*	Response regulator; flagellar bias modulation
fliZ	**7.5, 11.2**	–, –	2.8, 3.7	–, –	*fliBC–ylxL*	Required for flagellar formation; SS; 1 TMS
fliP	–, 1.9	–, –	–, –	–, –	*fliBC–ylxL*	Required for flagellar formation; 5 TMS
fliQ	–, 1.1	–, –	–, –	–, –	*fliBC–ylxL*	Required for flagellar formation; 2 TMS
fliR	3.6, –	–, –	–, –	–, –	*fliBC–ylxL*	Required for flagellar formation; SS; 7 TMS
flhB	2.0, –	–, –	–, –	–, –	*fliBC–ylxL*	Flagellar formation protein; 4 TMS
flhA	**5.4, 4.7**	–, 1.8	2.0, 1.1	–, –	*fliBC–ylxL*	Flagellar formation protein; SS; 7 TMS
flhF	**7.2, 7.9**	–, –	**3.3, 3.9**	–, –	*fliBC–ylxL*	Flagella-associated protein
ylxH	–, 3.7	–, –	–, 1.2	–, –	*fliBC–ylxL*	Similar to flagellar biosynthesis switch protein; 1 TMS
cheB	–, –	–, –	–, –	–, –	*fliBC–ylxL*	MCP-Glu methylesterase/response regulator-like
cheA	–, –	–, –	–, –	–, –	*fliBC–ylxL*	Chemotactic signal kinase; 1 TMS
cheW	**4.9, 3.5**	–, –	1.1, 1.2	–, –	*fliBC–ylxL*	CheA activity modulator in response to attractants
cheC	–, –	–, –	–, –	–, –	*fliBC–ylxL*	Inhibition of CheR-mediated methylation of MCPs
cheD	**3.6, 3.1**	–, –	1.7, 1.3	0.6, 0.5	*fliBC–ylxL*	Required for methylation of MCPs by CheR
sigD	**3.3, 3.1**	–, –	1.4, 1.3	–, 0.4	*fliBC–ylxL*	RNA polymerase sigma-28 factor (sigma-D)
ylxL	6.6, 4.9		1.7, 1.6	0.4, 0.3	*fliBC–ylxL*	Unknown function, SS
flgM	3.3, 2.1	2.8, 4.8	–, –	–, –	*flgM-****yvyG-flgK-flgL***	Flagellin synthesis regulatory protein
yvyG	**4.2, 4.5**	–, 1.9	1.7, 0.9	–, –	*flgM-****yvyG-flgK-flgL***	Similar to flagellar protein
flgK	**6.1, 5.5**	1.9, 3.5	2.0, 0.9	0.6, 0.6	*flgM-****yvyG-flgK-flgL***	Flagellar hook-associated protein 1
flgL	**4.5, 4.0**	2.5, 2.3	1.2, 0.9	0.7, 0.5	*flgM-****yvyG-flgK-flgL***	Flagellar hook-associated protein 3
flhO	**3.0, 3.5**	1.2, 4.7	2.7, 1.0	1.1, 1.3	***flhO****-flhP*	Flagellar basal-body rod protein
flhP	1.7, 1.8	1.7, 2.1	1.2, 0.6	1.2, 0.7	***flhO****-flhP*	Flagellar hook-basal body protein
fliD	**6.6, 7.2**	–, 4.6	6.1, 1.2	2.5, 0.7	***fliD-fliS****-fliT*	Flagellar hook-associated protein 2
fliS	**3.7, 5.2**	–, 3.9	2.8, 1.0	1.5, 0.7	***fliD-fliS****-fliT*	Flagellar protein
fliT	2.6, 2.8	1.2, 2.2	2.1, 0.9	1.0, 0.7	***fliD-fliS****-fliT*	Flagellar protein
hag	**32.1, 30.0**	**6.3, 4.8**	**4.0, 10.0**	–, –	***hag***	Flagellin protein
lipA	5.3, 5.1	0.4, 0.5	7.5, 15.6	0.6, 1.5	***lipA***	Probable lipoic acid synthetase
lytA	1.9, 2.1	–, –	–, –	–, –	*lytA-****lytB****-lytC*	Secretion of major autolysin LytC
lytB	**3.8, 3.4**	–, –	**4.2, 5.3**	2.0, 1.8	*lytA-****lytB****-lytC*	Modifier protein of major autolysin LytC; SS
lytC	3.9, 2.7	1.3, 1.3	4.0, 1.5	1.3, 0.8	*lytA-****lytB****-lytC*	Major autolysin CWBP49; SS
lytF	**8.0, 5.5**	**3.4, 3.0**	3.0, 1.7	–, –	***lytF***	Major autolysin CWBP49'
mcpA	**10.0, 6.6**	–, –	2.9, 1.2	–, –	***mcpA***	Methyl-accepting chemotaxis protein; SS
mcpB	**4.7, 4.7**	3.8, 2.4	2.2, 1.4	1.8, 0.7	***mcpB***	Methyl-accepting chemotaxis protein; SS; 2 TMS
mcpC	**4.7, 5.1**	**5.1, 5.8**	1.0, 0.4	1.1, 0.5	***mcpC***	Methyl-accepting chemotaxis protein; SS; 2 TMS
motA	**6.5, 5.1**	–, –	2.8, 1.0	–, –	***motA-motB***	Motility protein A; SS; 3 TMS
motB	**8.2, 7.0**	1.8, 5.4	2.4, 0.8	0.5, 0.6	***motA-motB***	Motility protein B; 1 TMS
pckA	**4.2, 3.8**	1.0, 0.1	1.5, 5.2	0.4, 0.1	***pckA***	Phosphoenolpyruvate carboxykinase; 1 TMS
srfAA	**4.9, 3.7**	**3.2, 35.9**	0.1, 0.01	0.1, 0.4	***srfAA-srfAB-srfAC-srfAD***	Surfactin synthase; 4 TMS
srfAB	**7.4, 5.7**	**3.1, 43.2**	0.1, 0.1	0.01, 0.4	***srfAA-srfAB-srfAC-srfAD***	Surfactin synthase; 4 TMS
srfAC	**6.2, 4.1**	**4.3, 25.0**	0.1, 0.1	0.1, 0.7	***srfAA-srfAB-srfAC-srfAD***	Surfactin synthase; 1 TMS
srfAD	**8.7, 3.5**	**3.1, 34.3**	0.1, 0.1	0.04, –	***srfAA-srfAB-srfAC-srfAD***	Surfactin synthase

Table 2 (Contd.)

Gene[a]	Ratio[b]				Transcriptional organization[c]	Product function[d]
	wt/hy (log)	wt/hy (stat)	log/stat (wt)	log/stat (hy)		
sspB	**5.8, 7.9**	1.7, 0.8	**3.3, 6.9**	1.0, 0.7	***sspB***	Small acid-soluble spore protein
tagC	**3.0, 5.8**	1.1, –	2.6, 5.7	1.0, 1.8	***tagC***	Involved in polyglycerol phosphateteichoic acid biosynthesis
wapA	**7.0, 10.6**	–, 7.9	**8.4, 3.6**	1.3, 1.0	***wapA-yxxG***	Cell wall-associated protein precursor; SS
yxxG	**14.1, 11.9**	–, –	**9.4, 3.4**	2.5, –	***wapA-yxxG***	Unknown function
xylA	**7.7, 8.8**	**77.8, 117.1**	0.4, 0.2	3.1, 2.7	***xylA-xylB***	Xylose isomerase
xylB	**7.5, 6.7**	**17.4, 29.3**	1.4, 0.5	3.1, 2.4	***xylA-xylB***	Xylulose kinase
ybfN	**7.3, 15.0**	1.1, 1.0	**4.1, 11.5**	0.6, 0.8	***ybfN***	Unknown function
ydjK	**9.4, 10.4**	–, –	**8.0, 19.2**	1.0, 2.8	***ydjK***	Similar to sugar transporter; SS; 12 TMS
ydzF	**5.7, 8.6**	–, –	2.6, 2.9	–, –	***ydzF***	Unknown function
yfiJ	**3.9, 7.6**	1.4, 1.3	**3.4, 7.6**	1.2, 1.3	***yfiJ-yfiK***	Similar to sensor histidine kinase; SS; 4 TMS
yfiK	–, 1.5	–, –	–, –	–, –	***yfiJ-yfiK***	Similar to two-component response regulator
yfkC	**3.1, 6.5**	–, –	**3.8, 4.9**	2.0, –	***yfkC***	Unknown function; SS; 3 TMS
yfkL	**3.3, 3.5**	–, –	1.4, 2.0	–, –	***yfkL***	Similar to resistance protein; SS; 10 TMS
yfmO	**6.2, 14.4**	–, 1.7	**5.3, 13.0**	1.3, 1.5	***yfmO***	Similar to multidrug-efflux transporter; SS; 11 TMS
yfmT	**8.6, 7.5**	–, –	2.0, 2.5	–, –	***yfmT-yfmS***	Similar to benzaldehyde dehydrogenase
yfmS	**13.8, 10.1**	–, –	3.3, 2.3	–, –	***yfmT-yfmS***	Similar to methyl-accepting chemotaxis protein
yhcO	–, –	–, –	–, –	–, –	*yhcO-**yhcP***	Unknown function; SS
yhcP	**4.0, 3.1**	–, –	2.4, 1.0	–, –	*yhcO-**yhcP***	Unknown function
yhdN	**3.7, 8.2**	1.1, 0.9	2.6, 5.3	0.8, 0.6	***yhdN***	Similar to aldo/keto reductase
yhdY	**5.1, 10.9**	1.4, 1.1	**4.0, 17.5**	1.1, 1.8	***yhdY***	Unknown function; 5 TMS
yheF	5.2, 3.3	–, –	2.7, 1.1	–, –	***yheF**-yheG*	Unknown function
yheG	1.6, 1.2	1.1, 1.3	1.3, 1.0	0.9, 1.1	***yheF**-yheG*	Similar to calcium-binding protein
yhfE	**5.4, 11.2**	1.7, –	2.6, 6.5	0.8, –	***yhfE**-yhfF*	Similar to glucanase
yhfF	1.0, 0.7	1.0, 1.3	0.8, 0.2	0.7, 0.4	***yhfE**-yhfF*	Unknown function
yhxD	**3.7, 3.4**	2.1, 1.5	**3.3, 4.4**	1.9, 2.0	***yhxD***	Similar to ribitol dehydrogenase
yitA	**5.5, 5.7**	–, –	3.4, 1.9	–, –	***yitA**-yisZ*	Similar to sulfate adenylyltransferase
yisZ	1.5, –	1.9, 1.0	0.5, –	0.7, 0.5	***yitA**-yisZ*	Similar to adenylylsulfate kinase
ykvU	**6.6, 7.6**	1.1, 0.5	**4.2, 17.1**	0.7, 1.2	***ykvU**-ykvV*	Similar to spore cortex protein; SS; 10 TMS
ykvV	1.7, 2.2	–, –	1.6, 1.5	1.4, –	***ykvU**-ykvV*	Unknown function; SS
yloC	**4.6, 9.3**	1.4, 0.9	**6.9, 15.8**	2.1, 1.5	***yloC***	Unknown function; SS
ynaJ	–, –	**15.4, 13.3**	0.2, –	–, –	***ynaJ-xynB***	Similar to H + -symporter; 10 TMS
xynB	1.0, 0.9	**5.4, 6.2**	0.2, 0.1	1.2, 1.0	***ynaJ-xynB***	Xylan beta-1.4-xylosidase
ynzD	**5.0, 7.0**	1.2, 1.2	**3.1, 6.8**	0.7, 1.2	***ynzD***	Unknown function
yobL	0.9, 0.7	1.1, 1.3	0.7, 0.6	0.9, 1.1	*yobL-**yobK***	Unknown function
yobK	**7.5, 12.6**	–, –	**3.7, 4.2**	–, –	*yobL-**yobK***	Unknown function
yolA	**9.8, 5.8**	–, –	3.4, 1.9	–, –	***yolA**-yolB*	Unknown function; SS
yolB	–, –	–, –	–, –	–, –	***yolA**-yolB*	Similar to phage-related protein
yonS	**6.4, 3.8**	–, –	1.9, 1.3	–, –	***yonS***	Putative lipoprotein
yosX	3.8, –	–, –	1.5, –	–, –	*yosX-**yosZ***	Unknown function
yosZ	**3.8, 3.2**	–, –	1.4, 1.1	–, –	*yosX-**yosZ***	Unknown function
yppD	–, –	–, –	–, –	–, –	*yppD-**yppE***	Unknown function
yppE	**4.9, 7.3**	–, –	1.7, 2.4	–, –	*yppD-**yppE***	Unknown function
yrzE	**4.1, 14.6**	–, –	**3.2, 4.9**	–, –	***yrzE***	Unknown function; 4 TMS
ysbA	–, –	–, –	–, –	–, –	*ysbA-**ysbB***	Unknown function; SS; 4 TMS
ysbB	**3.7, 3.9**	1.0, 1.0	2.7, 7.3	0.7, 1.9	*ysbA-**ysbB***	Unknown function; SS; 5 TMS
yscA	**3.5, 7.8**	–, –	**3.1, 3.8**	–, –	***yscA-yscB***	Unknown function; SS
yscB	**5.5, 9.7**	1.1, 1.0	**4.6, 8.0**	0.9, 0.8	***yscA-yscB***	Putative membrane lipoprotein
ysfC	0.9, 1.9	–, –	3.4, 2.5	3.5, 2.1	***ysfC-ysfD***	Similar to glycolate oxidase subunit
ysfD	**9.2, 3.5**	–, –	2.4, 2.5	–, –	***ysfC-ysfD***	Similar to glycolate oxidase subunit
yshA	–, –	–, –	–, –	–, –	*yshA-**yshB***	Unknown function
yshB	**4.7, 4.0**	–, –	3.2, 2.8	–, –	*yshA-**yshB***	Unknown function; SS; 2 TMS
ytbQ	**6.9, 14.2**	0.5, 1.1	12.3, 15.7	0.8, 1.2	***ytbQ***	Unknown function; SS
ytdA	**3.3, 4.9**	–, –	1.3, 1.7	–, –	***ytdA***	Similar to UTP-Glc-1-phosphate uridylyltransferase
yuaD	**3.3, 5.8**	–, –	2.2, 2.2	–, –	***yuaD***	Unknown function
yufO	**4.3, 8.1**	2.2, 2.2	2.1, 6.2	1.1, 1.7	***yufO-yufP**-yufQ*	Similar to ABC transporter
yufP	**10.1, 25.0**	–, –	**5.0, 11.5**	–, –	***yufO-yufP**-yufQ*	Unknown function; SS; 5TMS
yufQ	0.8, 1.0	1.3, 1.1	0.6, 0.7	1.0, 0.8	***yufO-yufP**-yufQ*	Unknown function; SS; 6 TMS
yurO	**4.5, 12.0**	1.4, 0.5	2.7, 5.1	0.9, 0.2	***yurO***	Similar to multiple sugar-binding protein
yutH	**4.5, 3.9**	2.1, 1.6	1.7, 2.5	–, –	***yutH***	Unknown function
yvzB	**35.5, 20.5**	–, 6.2	5.4, 2.5	–, –	***yvzB***	Similar to flagellin
ywcI	2.2, 1.8	**3.3, 3.7**	0.3, 0.3	0.4, 0.6	***ywcI***	Unknown function; SS; 1 TMS
ywgB	–, 1.2	–, –	–, –	–, –	*ywgB-**mmr***	Unknown function; SS

Table 2 (Contd.)

Gene[a]	Ratio[b]				Transcriptional organization[c]	Product function[d]
	wt/hy (log)	wt/hy (stat)	log/stat (wt)	log/stat (hy)		
mmr	**3.2, 3.9**	–, –	2.0, 2.4	–, –	*ywgB-**mmr***	MethylenomycinA resistance protein; SS; 13 TMS
ywlC	**4.5, 5.2**	0.3, 0.3	**5.9, 6.7**	0.4, 0.4	***ywlC***	Unknown function
ywnA	2.7, –	–, –	1.6, –	–, –	*ywnA-**ywnB***	Unknown function
ywnB	**5.0, 3.9**	1.3, 1.0	2.6, 3.1	0.7, 0.8	*ywnA-**ywnB***	Unknown function
ywpD	**11.9, 12.4**	1.1, 1.2	**7.1, 11.8**	0.7, 1.1	***ywpD***	Similar to two-component sensorhistidine kinase; SS; 1 TMS
ywtD	**9.9, 6.3**	–, –	3.2, 2.1	–, –	***ywtD***	Similar to murein hydrolase; SS
yxkC	**22.5, 22.9**	–, –	**18.4, 7.6**	–, –	***yxkC***	Unknown function; 1 TMS
yydA	**5.1, 5.9**	1.1, 1.5	**5.9, 12.1**	1.2, 3.0	***yydA***	Unknown function

[a]Gene names highlighted in *bold* indicate lower expression in *B. subtilis degU32*(Hy) as revealed by proteome analysis

[b]The calculated expression level ratios are shown for the two independent macroarray experiments performed (– indicates that the signals were below the significance threshold). log, exponential phase of growth; stat, stationary growth phase. Ratios shown in *bold* indicate significant up-regulation, defined as a ≥3-fold change in mRNA levels in both macroarray experiments

[c]Gene names depicted in *bold* indicate significant down-regulation of these loci in the exponential or the stationary growth phase in *B. subtilis degU32*(Hy). Genes identified by Ogura et al. (2001) as putative members of the DegU regulon are *underlined*

[d]SS and TMS indicate the presence of signal sequences and transmembrane segments, respectively, as predicted by *in silico* analysis of the encoded proteins

strains. The *wapA* gene encodes a cell wall-associated protein precursor, and is cotranscribed with the promotor-distal *yxxG* gene as a bicistronic 8.0-kb *wapA-yxxG* mRNA. The Northern blot verified the down-regulation of the *wapA-yxxG* mRNA in the mutant strain in the exponential growth phase, where only a faint signal was obtained. In the stationary phase, very weak signals were detected in both strains.

The *xylB* gene encodes xylulose kinase, and is co-transcribed with *xylA* (xylose isomerase) as a bicistronic 3.0-kb mRNA. According to the transcriptome analysis, there about 7-fold less *xylB* mRNA is present in the *degU32*(Hy) mutant than in the wild type in the exponential phase, and there is 20- to 30-fold less in stationary phase (Table 2). The DegU-P dependent negative regulation of the *xylAB* operon has not been noted previously, and was confirmed by the Northern analysis.

Discussion

The analysis of the global gene expression profile of wild-type *B. subtilis* and the *degU32*(Hy) mutant by means of a combination of proteomic and transcriptomic approaches has confirmed most of the previously identified members of the DegS-DegU regulon. The *degU32*(Hy) mutant contains high levels of phosphorylated DegU, resulting in the pleiotropic "hy" phenotype. Besides overproduction of degradative extracellular enzymes, this phenotype includes impaired motility and defects in the development of transformation competence, as well as filamentous morphology. Comparison of the gene expression patterns of the wild type and the *degU32*(Hy) mutant in LB medium allowed the identification of several new potential target genes for DegU-P. Interestingly, DegU-P activated genes were more strongly expressed in the mutant strain either during exponential growth or in the stationary phase. Of the proteins of known function that are up-regulated in the mutant, the majority represented extracellular degradative enzymes.

Several genes (*bglC*, *bglS*, *bpr*, *ggt*, *mpr*, *pelB*, *vpr*, *yfkN*, *ynfF*, *yurI* and *ywaD*) whose protein products were present in larger amounts in the extracellular proteome of the *degU32*(Hy) mutant strain could only be identified as belonging to the DegS-DegU regulon by the proteomic approach. Of the genes identified by the extracellular proteome analysis, only *amyE*, *aprE* and *nprE* exhibited significantly higher levels of mRNA. Moreover, *bglS*, *yurI* and *ywaD* specified nearly three times as much mRNA in the mutant, as revealed by the macroarray analysis. Most probably, the extracellular proteins, which are much more stable than their short-lived mRNAs, accumulate to higher levels in the mutant, thus explaining why induction ratios are higher at the protein level than the mRNA level. In contrast, *csn*, which codes for an extracellular enzyme, was clearly DegU-P dependent according to the array analysis, but could not be assigned by the proteomic approach. Possibly, this can be attributed to the fact that not all up-regulated proteins have been identified so far (Fig. 1A).

Negative regulation of many DegU-P activated genes by additional factors during exponential growth was reflected by the significant stationary-phase induction observed in the *degU32*(Hy) mutant for *amyE*, *aprE*, *bglS*, *mpr*, *nprE*, *vpr*, *yfkN*, *ynfF*, *yurI* and *ywaD*. These genes were induced on the mRNA and the protein level in stationary phase. The *aprE* gene exhibited the strongest mRNA induction ratio (around 150-fold), which was surprising in view of the small increase (around 2-fold) observed on the protein level. The AprE protein was already present as a strong spot in the exponential growth phase in the *degU32*(Hy) mutant,

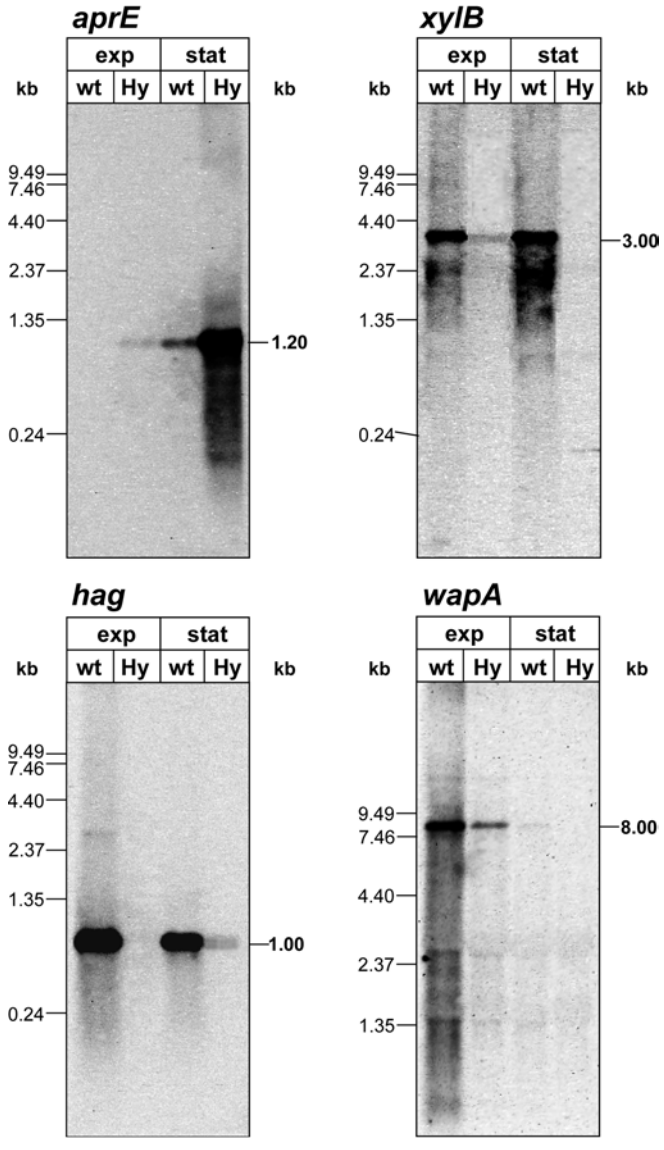

Fig. 3 Northern analysis of selected DegS-DegU regulated genes. RNA was isolated from wild-type *B. subtilis* 168 (wt) and *B. subtilis* *degU32*(Hy) grown in LB medium. Exponentially growing cells were harvested at an OD_{540} of 0.4 (exp) and 1 h after the transition into stationary phase at an OD_{540} of 3.5 (stat). The following amounts of total RNA were applied per lane: *xylB* and *wapA*, 5 μg; *aprE* and *hag*, 2.5 μg. The gene-specific probes used in the different experiments are indicated. The transcript sizes marked on the *right* were determined by comparison with an RNA size marker (Gibco BRL, Eggenstein, Germany); band positions are depicted on the *left*

despite the low level of *aprE* mRNA detected, which was verified by Northern hybridization. The AprE protein might be exceptionally stable, resulting in extracellular accumulation of large amounts of this protein starting in the exponential growth phase.

As expected, the group of genes found to be down-regulated in the *degU32*(Hy) mutant included those of the σ^D regulon and the *srfAAABACAD* operon which encodes the surfactin synthetase and the competence regulatory factor ComS (DSouza et al. 1994). It is known that competence development is nearly abolished in the *degU32*(Hy) mutant in two ways. On the one hand, accumulation of DegU-P in the mutant causes low amounts of unphosphorylated DegU protein which is in the turn required for the expression of the competence transcription factor ComK. On the other hand, the phosphorylated form of DegU acts as a repressor of *comS*, which encodes an essential component of the competence activation pathway (Msadek et al. 1993). However, expression of the late competence genes was not significantly affected according to our analysis.

The microarray analysis published by Ogura et al. (2001) also led to the identification of several putative new members of the DegS-DegU regulon. Altogether 67 genes organized in 32 (partially putative) transcriptional units were postulated to be regulated positively, and 48 genes organized in 27 (partially putative) transcriptional units were proposed to be regulated negatively by the DegS-DegU two-component system. Our study postulates 43 positively regulated genes organized in 34 transcriptional units and 97 negatively regulated genes organized in 64 transcriptional units. Altogether, 13 positively regulated transcriptional units and 9 negatively regulated transcriptional units were predicted by both studies. In addition to the positively regulated genes described by Ogura et al. (2001) and in earlier studies, our analysis predicts 12 new members of this regulon. The 13 extracellular proteins regulated by DegU-P that were identified by comparison of the wild type and the *degU32*(Hy) mutant by the proteomic approach were recently described by Antelmann et al. (2001). The list of DegU-P regulated genes published by Ogura et al. (2001) exhibited the strongest similarity to our list of genes significantly up- or down-regulated in the *degU32*(Hy) mutant in the exponential growth phase (Tables 1 and 2). In this context, the methodological differences between the two studies must be emphasized: Ogura et al. (2001) induced the overexpression of unphosphorylated DegU in Schaeffer's sporulation medium by the addition of IPTG in the exponential growth phase and harvested the cells 2 h later (corresponding to approximately 30 min after the transition into stationary phase). In our study, wild-type *B. subtilis* and the *degU32*(Hy) mutant, which produces a highly stable form of the phosphorylated DegU protein, were compared in LB medium during exponential growth and 1 h after the transition point. Therefore, the differences between the results of the two studies may be due mainly to the differences in experimental conditions.

A large percentage of the DegU-P regulated proteins with unknown functions are predicted to have transmembrane sequences and/or signal sequences according to in silico analyses (Tables 1 and 2). This finding is in agreement with the fact that many previously known DegU-P dependent genes code for extracellular proteins. Furthermore, this finding might indicate that activation of the DegS-DegU regulon results in alterations in membrane protein composition, possibly as a consequence of the induced synthesis of transport systems.

Suitable follow-up experiments are necessary to verify this assumption.

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