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Proteome Analysis of *Bacillus subtilis* When Overproducing Secretory Protein

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Abstract *Bacillus subtilis* and related *Bacillus* species are frequently used as hosts for the mass production of recombinant proteins. Accordingly, this study examined the cellular response of *B. subtilis* to the overexpression of a soluble secretory protein. As such, the lichenase derived from *B. cereus* was overexpressed in *B. subtilis*, initially localized in the cytoplasm as a mature form and then secreted into the medium. Thereafter, the proteome of *B. subtilis* was analyzed using 2D electrophoresis and MALDI-TOF mass spectrometry. The expression of several heat-shock proteins, such as *dnaK* and *groEL*, was increased under this condition. In addition, manganese superoxide dismutase and NADH dehydrogenase were also upregulated in the lichenase-secreting *B. subtilis*. Therefore, it was concluded that the transient accumulation of a secreted protein in *B. subtilis* before secretion acted as a stress on the cell, which in turn induced the expression of various protective proteins.

Key words: Proteome, *Bacillus*, lichenase, secretion

There have been several previous studies regarding the physiological responses of the Gram-negative bacterium *Escherichia coli* to the overproduction of recombinant proteins [24, 4, 18]. As a result, it has been demonstrated that *E. coli* cells respond to the overproduction of recombinant proteins by increasing the expression of chaperone molecules such as GroEL, DnaK, and Tig, and by decreasing the levels of ribosomal proteins such as S6 and L9 [21, 12, 20]. *E. coli* cells involved in the overproduction of recombinant proteins have also shown a significant yet transient increase in the mRNA levels of heat-shock genes such as *lon* and *dnaK* [12]. The heat-shock-like response during heterologous protein expression also often results in an elevation of intracellular proteolytic activities [15, 10]. Strong expression systems frequently result in an accumulation of recombinant proteins in the form of inclusion bodies, and it has been demonstrated that the composition of inclusion bodies containing insoluble recombinant proteins can be remarkably homogeneous [21, 12]. Besides the fragmentation of heterologous proteins, host stress proteins, like DnaK, GroEL, IbpA, IbpB, and OmpT, have also been found in inclusion bodies from *E. coli*.

Gram-positive *Bacillus* species have many features for diverse biotechnological applications, especially for the industrial production of heterologous proteins [11, 23], plus the entire genome of *B. subtilis* has already been sequenced [17]. The most important biotechnological feature of *Bacilli* is their ability to secrete a multitude of proteins into the growth medium [3, 2]. Although members of the genus *Bacillus* are preferred hosts for industrial protein production [23, 5], detailed reports on the physiology of the Gram-positive bacteria during the overproduction of recombinant proteins are relatively few [13]. Accordingly, the aim of this study was to gain a comprehensive understanding of the cellular response to the overproduction of secretory heterologous proteins in *B. subtilis*. Thus, to examine the cellular response and adaptation of this microorganism during the overexpression of a soluble secretory protein, we analyzed the changes in the proteome level. In particular, the cytoplasmic proteomes of *Bacillus* in the overproduction process were profiled and the results compared with those obtained with control cells.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions

pBE1 containing the pBR322 origin, pUB origin, and the BJ27X promoter was used as the expression vector. *B. subtilis* RM125 (pBE1) was used as the control cell and *B.*

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subtilis RM125 (pBLC771) as the expression host for the lichenase. The *Bacillus* strains were cultured in a Luria broth complex medium at a constant temperature of 37°C until the early stationary phase. Cell growth was monitored by measuring the OD_{600} throughout the culture, and kanamycin (typically 20 mg/l) was included in all the agar plates for initial selection pressure, and also in the liquid medium.

Sample Preparation

The *Bacillus* intracellular proteins were concentrated by TCA precipitation (final 10%) of the lysates prepared by disruption using a French-press (1,500–1,800 lb) (Thermo Spectronic, NY, U.S.A.). The extracellular proteins from the culture supernatant were also concentrated by TCA precipitation followed by ethanol washing. The concentration of protein was determined using a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) and bovine serum albumin as the standard. The proteins were stored at -70°C, then re-dissolved in a rehydration solution before electrophoresis.

Two-Dimensional Gel Electrophoresis

For the first-dimensional separation, pH 4–7 linear immobilized pH gradient (IPG) gel strips (13 cm; Amersham Pharmacia Biotech, U.K.) were rehydrated overnight with a rehydration solution containing 250 mg of the protein in an IPGphor strip holder covered with cover fluid. A three-phase program was then used for the isoelectric focusing. The first phase was at 1,000 V for 1 h, the second phase was set at 2,000 V for 2 h, and the third phase was a linear gradient from 2,000 V to 8,000 V for 14 h. After the first-dimensional isoelectric focusing, the IPG strips were recovered and equilibrated with an SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 2% SDS, 30% glycerol, and 0.05% bromophenol blue) for 15 min. The second-dimensional separation was then carried out in 12% SDS-PAGE gels (16×20 cm; without stacking gels) at 4°C. The electrophoresis was performed with a constant current setting at 60 mA/gel for 6 h until the bromophenol blue reached the bottom of the gels. Meanwhile, the protein spots were visualized by silver staining. For the subsequent MALDI-TOF analysis, the silver was removed from the gel using chemical reducers [7], and in-gel trypsin digestion of the proteins performed as described in a previous report [14].

MALDI-TOF Mass Spectrometry and Database Search

All the mass spectrometric analyses were performed using a PerSeptive Biosystems MALDI-TOF Voyager DE-RP mass spectrometer (Framingham, MA, U.S.A.) operated in the delayed extraction and reflector mode. The peptide mixtures were analyzed using a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid, and the program PEPTIDENT from ExPASY used for the database searches.

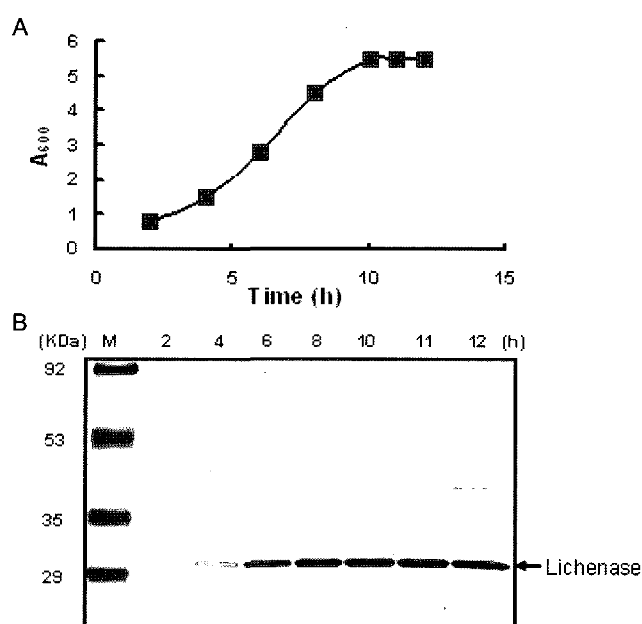

Fig. 1. Overexpression of lichenase in *B. subtilis* RM125.
A. Growth curve of *B. subtilis* RM125. **B.** SDS-PAGE analysis of overexpressed lichenase.

RESULTS

The proteins to be analyzed were prepared from the control and lichenase-overproducing *Bacillus* cells during the early stationary phase (Fig. 1). The overexpressed lichenase was successfully secreted into the culture medium and its amount steadily increased in a time-dependent manner (Fig. 1). The intracellular and extracellular proteins from both strains were concentrated by TCA precipitation and then separated by 2-DE (pI 4–7; Figs. 2, 3). The 2-DE

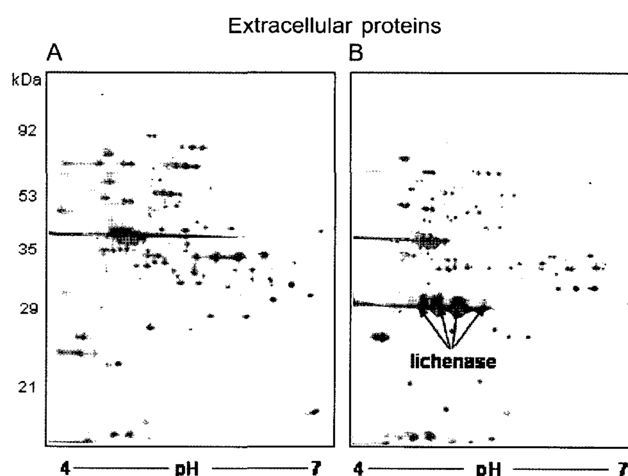

Fig. 2. 2D pattern of extracellular proteins from (**A**) *B. subtilis* RM125 harboring pBE1 vector only, and (**B**) *B. subtilis* RM125 overproducing lichenase (indicated by arrows).

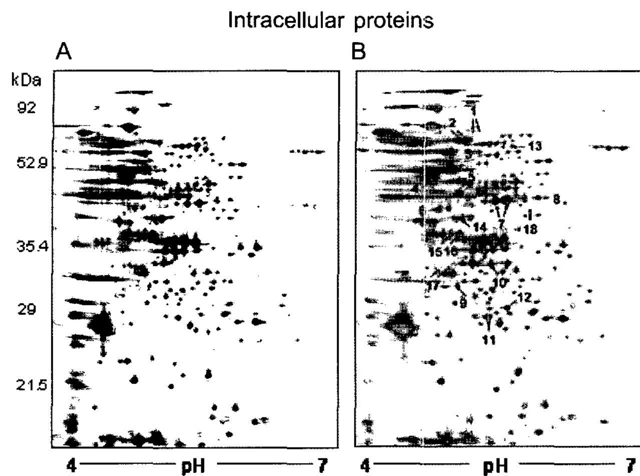

Fig. 3. 2D patterns of intracellular proteins from (**A**) *B. subtilis* RM125 harboring pBE1 vector only, and (**B**) *B. subtilis* RM125 overproducing lichenase.

pattern of the extracellular proteins clearly showed that lichenase had been secreted very efficiently, and four major protein spots with the same molecular weight (~30 kDa) yet with different pI values were confirmed as lichenase by a MALDI-TOF analysis (Fig. 2B). Although the same protein amounts were applied for the 2D electrophoretic (2DE) analysis, the intensities of the other protein spots in the lichenase-overproducing strain were consistently lower, owing to the presence of the overexpressed lichenase (Fig. 2). Therefore, for the 2DE analysis of the intracellular proteins, the amount of protein obtained from the lichenase-overproducing cells was re-adjusted accordingly, and then the overall 2DE pattern of the intracellular proteins looked similar to that for the control cells (Fig. 3).

The 2DE analysis of the intracellular proteins revealed that several proteins were up- or downregulated by the overexpression of lichenase (Fig. 4), and a mass analysis by MALDI-TOF identified 12 upregulated and 6 downregulated proteins. When compared with the control, the levels of the proteins involved in responding to stress and the defense mechanism, such as DnaK, Gsp30 (general stress protein 30), manganese superoxide dismutase (Mn-SOD), and NADH dehydrogenase, were significantly increased in the lichenase-overproducing strain. In addition, the protein level of the ABC transport ATP-binding YdiF was also upregulated. Furthermore, the levels of the proteins involved in cellular metabolism (e.g., glyceraldehyde-3-phosphate dehydrogenase, enolase, aldolase, AroA, alpha-acetolactate decarboxylase, and phosphoadenosine phosphosulfate reductase) were all increased in the lichenase-

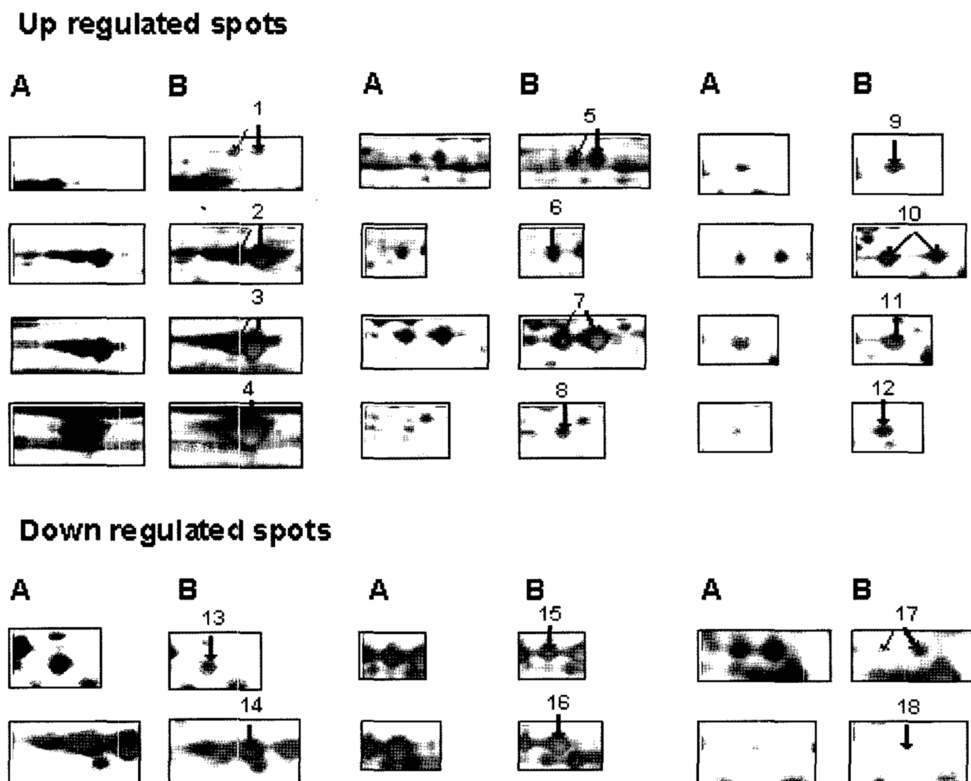

Fig. 4. Close-up analysis of areas shown in Fig. 3, demonstrating that several spots are up- or downregulated by overproduction of lichenase.

A, *B. subtilis* RM125 harboring pBE1 vector only. **B**, *B. subtilis* RM125 overproducing lichenase.

Table 1. List of upregulated proteins.

Spot no.	Accession no.	Protein identification	pI[a]	M_r[a]	Coverage[b]	Match[c]
Metabolism						
4	P37869	Enolase	4.7	46.6	19	7
7	P09124	Glyceraldehyde-3-phosphate dehydrogenase 1	5.3	35.7	33	12
8	Q04777	Alpha-acetolactate decarboxylase	4.8	28.8	15	5
9	P39912	AroA(G) protein	5.5	39.5	19	8
10	P13243	Probable fructose-bisphosphate aldolase 1	5.2	30.4	29	6
12	P94498	Phosphoadenosine phosphosulfate reductase	5.6	30	24	4
Stress Response						
2	P17820	Chaperone protein dnaK (HSP70)	4.8	65.9	6	4
3	P42974	NADH dehydrogenase	5	54.9	26	12
6	P42101	General stress protein 30 (GSP30)	4.8	36.4	15	5
11	P54375	Superoxide dismutase [Mn]	5.2	25.2	20	3
Transport/Binding Proteins						
1	O05519	Hypothetical ABC transporter ATP-binding protein ydiF	5.1	73.5	17	4
Unknown						
5	P54542	Hypothetical protein yqjE	5	39.7	27	8

[a]Theoretical value using ExPASY tool.
[b]Percent coverage obtained for the sequence of the identified protein.
[c]Number of peptide sequence matches.

overproducing strain (Table 1), whereas the proteins involved in protein synthesis were upregulated. In contrast, the expression of six proteins was decreased in the lichenase-overexpressing strain (Table 2). Interestingly, flagellin involved in cell motility and chemotaxis was downregulated. In addition the levels of four metabolic proteins, 2-oxoisovalerate dehydrogenase, malate dehydrogenase, LolI, and keto-acid reductoisomerase, were decreased in the lichenase-overexpressing strain. YrkE, whose function is still unknown, was downregulated.

DISCUSSION

The cellular response during the overproduction of recombinant proteins in the Gram-negative bacterium *E. coli* has already been well studied, yet little is known in this regard in other microorganisms. Recently, a few studies on the cellular response of *Bacillus* strains to the overproduction of foreign proteins have been attempted, as understanding the cellular response to the overexpression of heterologous proteins on a molecular level is crucial to improve the productivity of secretory recombinant proteins in *Bacilli*. Overexpression experiments with *E. coli* have revealed that strong expression systems often cause a decrease in the expression of ribosomal genes [22, 12]. However, in this study, we did not observe any change in the expression of ribosomal proteins, at least on the proteome level. Instead, as previously shown in *E. coli* and *B. subtilis* [13], the level of DnaK, a class I heat-shock protein, was increased in the lichenase-overproducing strain. In a previous experiment on the overexpression of the basic fibroblast growth factor (bFGF) in *E. coli*, the normal cellular stress response caused by the high inducing temperature was strongly amplified by heterologous protein synthesis, which included soluble and inclusion body form proteins. In particular, the synthesis of proteins involved in translation and protein folding was affected by the

Table 2. List of downregulated proteins.

Spot no.	Accession no.	Protein identification	pI	M_r	Coverage	Match
Mobility and Chemotaxis						
13	P02968	Flagellin	5	32.6	24	4
Metabolism						
14	P37940	2-Oxoisovalerate dehydrogenase alpha subunit	5	36.3	20	8
15	P49814	Malate dehydrogenase (VEG69)	5	33.5	35	8
16	P42419	LolI protein	4.9	31.7	23	5
17	P37253	Ketol-acid reductoisomerase	5.5	37.5	35	10
Unknown						
18	P54432	Hypothetical protein yrkE	6	17.8	17	4

overproduction of the heterologous protein [22]. Consequently, this finding demonstrates that the transient accumulation of a secretory protein in *Bacillus* strains before secretion can induce a heat-shock-like response similar to that found in *E. coli*. It has also been reported that the presence of the *B. subtilis hrcA* gene and an overproduction of insoluble proteins in *E. coli* mediate the induction of a transcriptional *hrcA-bgaB* fusion containing the CIRCE operator [19]. This induction is also inversely correlated with the cellular amount of the chaperonin GroE (GroEL/ES). As such, it has been proposed that the increased level of non-native proteins causes a titration of GroE, which in turn prevents reactivation of the HrcA repressor and consequently increases the expression of class I heat-shock genes. In addition to DnaK, the levels of Mn-SOD, the general stress protein 30, and NADH dehydrogenase are also increased. Furthermore, it is well-established that the overproduction of foreign proteins (usually accumulated within the cells) is stressful to the host cell [13, 22, 19]. Thus, our findings that stress proteins were also upregulated in *Bacillus* overproducing lichenase not only confirm these previous results but also indicate that even the overexpression of secretory proteins, which are not accumulated intracellularly, could be stressful to the host cells.

In *E. coli*, the protease La, encoded by the *lon* gene, is generally supposed to be the primary protease involved in the degradation of aberrant proteins and recombinant proteins [8, 9]. In *B. subtilis*, the major cytoplasmic protease appears to be the Clp protease consisting of the peptidase subunit ClpP and ATPase subunit ClpC [6, 16]. It has also been shown that Clp proteases are mainly involved in the proteolysis of misfolded proteins that can occur after heat shock. Unexpectedly, in this study, we could not detect any upregulation of the Clp protease (or any kind of protease), implying that the overexpression of a secretory protein does not necessarily induce the proteolytic system, which differs significantly from the authentic heat-shock response that involves the accumulation of proteins inside the cells. Therefore, the overexpression of the protein caused stress to the cell and induced class I heat-shock proteins (DnaK, GroEL), whether expressed in a soluble or secretory form, or made inclusion bodies. In the case of the protein making inclusion bodies, class II heat-shock proteins (ClpC, ClpP) were also induced. In addition, this study showed that other stress response proteins (NADH dehydrogenase, Mn-SOD) were also induced by the overexpression of a secretory protein.

The finding that the levels of glycolytic enzymes (enolase, GAPDH, and fructose biphosphate aldolase) were increased may have been due to the increased requirement of energy production for the synthesis of foreign proteins. Similarly, the upregulated chorismate mutase could be accounted for by the increased requirement for aromatic amino acids in the overproducing cells. In contrast, ketol acid reductoisomerase for the synthesis of valine and isoleucine was downregulated in the same cells expressing lichenase. Possibly, the upregulation of chorismate mutase was specifically required for the overproduction of lichenase, which contains a number of aromatic amino acids.

The acetoin-producing enzyme alpha-acetolactate decarboxylase was also upregulated in the lichenase-overproducing cells. A previous study showed that the accumulation of acetoin in *E. coli* enhances recombinant protein production [1]. When the *alsS* gene from *Bacillus subtilis* encoding acetolactate synthase is introduced into *E. coli*, this newly introduced heterologous enzyme modifies the glycolytic fluxes by redirecting excess pyruvate away from acetate to acetolactate. The acetolactate is then converted to a non-acidic and less harmful by-product, acetoin, which appears in the broth. Furthermore, comparative fermentation studies have shown that the reduction in acetate accumulation leads to a significant improvement of recombinant protein production [1].

The identification of protein spots in a 2D gel is currently a limiting step in proteomic research. Nonetheless, even though we were only able to analyze 18 proteins that showed different expression patterns on the 2D gel, a number of physiological changes caused by the overexpression of lichenase could still be explained. As the number of identifiable protein spots increases, we will be able to understand global physiological changes under various environmental conditions, and this information can be utilized in relation to protein production and the construction of various databases regarding metabolism.

In summary, the present results demonstrated that the cellular response of the Gram-positive bacterium *B. subtilis* to the overproduction of a secretory protein, at least on a proteome level, was similar to the heat-shock-like response observed in the Gram-negative bacterium *E. coli*.

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