SacY, a Transcriptional Antiterminator from *Bacillus subtilis*,
Is Regulated by Phosphorylation In Vivo†

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SacY antiterminates transcription of the *sacB* gene in *Bacillus subtilis* in response to the presence of sucrose in the growth medium. We have found that it can substitute for BglG, a homologous protein, in antiterminating transcription of the *bgl* operon in *Escherichia coli*. We therefore sought to determine whether, similarly to BglG, SacY is regulated by reversible phosphorylation in response to the availability of the inducing sugar. We show here that two forms of SacY, phosphorylated and nonphosphorylated, exist in *B. subtilis* cells and that the ratio between them depends on the external level of sucrose. Addition of sucrose to the growth medium after SacY phosphorylation in the cell resulted in its rapid dephosphorylation. The extent of SacY phosphorylation was found to be proportional to the cellular levels of SacX, a putative sucrose permease which was previously shown to have a negative effect on SacY activity. Thus, the mechanism by which the sac sensory system modulates *sacB* expression in response to sucrose involves reversible phosphorylation of the regulator SacY, and this process appears to depend on the SacX sucrose sensor. The *sac* system is therefore a member of the novel family of sensory systems represented by *bgl*.

Sucrose induces the expression of two loci in *Bacillus subtilis*: the *sacB* gene, encoding levansucrase, and the *sacPA* operon, encoding a sucrose permease and a phosphosucrase (32, 48). Expression of these loci is controlled by transcriptional antitermination. Thus, in the absence of sucrose, transcription initiates constitutively and terminates at ρ-independent transcriptional terminators located between the respective promoters and the first structural genes; in the presence of sucrose, transcription proceeds through the putative terminators. The transcriptional antiterminators SacY and SacT are required for the sucrose-dependent readthrough of *sacB* and *sacPA*, respectively (7, 17, 18). Expression of *sacB* is negatively regulated by SacX, a sugar phosphotransferase-like protein (17). The *sacX* and *sacY* genes are contiguous and probably constitute an operon (5).

The *B. subtilis* antiterminators SacY and SacT highly resemble the BglG protein from *Escherichia coli*, both in sequence and in the mechanism of action (18, 47, 55). BglG prevents transcription termination at two terminators within the *bgl* operon by binding to the RNA chain and preventing the formation of the terminator structure (27). The RNA sequence recognized by BglG is highly conserved, and similar motifs which are found in the leader of both *sacB* and *sacPA* were suggested to be recognized by SacY and SacT, respectively (10). The activity of BglG is regulated by reversible phosphorylation (2, 3, 44) which, in turn, modulates the protein activity by controlling its dimeric state (4). Thus, BglG exists in the cell in two forms: an inactive, monomeric phosphorylated form and an active, dimeric nonphosphorylated form. Phosphorylation of BglG was recently shown to occur on a histidine residue (6) and was localized to His 208 (15). The state of BglG phosphorylation depends on the availability of β-glucosides; their addition to the growth medium leads to BglG dephosphorylation (3). The protein which functions as BglG kinase and phosphatase is BglF, an enzyme II of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), which is in charge of the transport and phosphorylation of β-glucosides (2, 3, 44). The way β-glucosides lead to *bgl* operon induction is by stimulating BglF to dephosphorylate BglG, thus allowing it to function as an antiterminator. In the absence of sugar, BglF inactivates BglG by phosphorylating it. BglG and BglF represent a novel family of systems which utilize a sensor and a regulator to transduce a signal from the cell surface to the transcription machinery (5).

Based on indirect evidence, regulation of *sacB* expression in *B. subtilis* seems to resemble *bgl* regulation in *E. coli*. Controlled readthrough of both systems is induced by sugars, sucrose and β-glucosides, respectively, and is positively regulated by homologous transcriptional antiterminators SacY and BglG, respectively. Moreover, while *bgl* expression is negatively regulated by BglF, the β-glucoside PTS permease, *sacB* is negatively regulated by SacX, a sucrose PTS permease homolog (9). Based on genetic studies, Crutz et al. (17) concluded that SacX suppresses *sacB* transcription in the absence of sucrose by inhibiting the antitermination activity of SacY. The general PTS proteins were also shown to negatively control *sacB* induction, most likely by inhibiting the function of SacY (17, 47). Based on these observations and on the striking similarity to the *bgl* system, a model was proposed which suggests that SacX is a sucrose sensor which is phosphorylated by the PTS general proteins and regulates SacY activity by phosphorylation according to sucrose availability in the medium (17).

To test this idea, we tested whether different forms of SacY, phosphorylated and nonphosphorylated, exist in *B. subtilis* cells. Two protein isoforms which differ in their charges were precipitated from extracts of cells induced for SacY expression. The isoforms were separated on two-dimensional (2-D) gels (isoelectric focusing in the first dimension and sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] in the second). Due to its sensitivity to phosphatase, the more acidic form was identified as the phosphorylated form of the protein. Our results indicate that SacY is regulated in vivo by reversible phosphorylation.

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† This work is dedicated to the memory of Michel Steinmetz.
The phosphorylation state of SacY depends on the availability of succinate, the inducer of the sac operon. Therefore, the level of phospho-SacY is expected to be lower in succinate-limited cells. In contrast, cells supplemented with 0.4% succinate as a carbon source showed a higher phosphorylation state of SacY. This observation is consistent with the finding that succinate induces the expression of the sac operon. Moreover, the phosphorylation state of SacY depends on the availability of the inducer, which is consistent with the regulation of the operon at the transcription level. The phosphorylation state of SacY is crucial for its function as an antiterminator, as it affects the ability of SacY to prevent the termination of transcripts in the sac operon. The phosphorylation state of SacY is also influenced by the availability of other nutrients, such as glucose, which can affect the expression of the sac operon and, consequently, the phosphorylation state of SacY. Therefore, the phosphorylation state of SacY is an important regulatory mechanism that affects the expression of the sac operon and the metabolism of the cell.
1). The ability of plasmid-encoded SacY to antiterminate transcription at the bgl terminator and enable lacZ expression in MA152 was tested by observing the color of the colonies containing these plasmids on MacConkey lactose plates and by measuring the β-galactosidase levels produced by the cells expressing them. As shown in Table 1, SacY behaved like BglG in its ability to allow for lacZ expression. This result indicates that not only are SacY and BglG similar in their sequences and activities but they also function in an identical manner.

**SacY is phosphorylated in the B. subtilis cell.** Based on the similarity between the sac system from *B. subtilis* and the bgl system from *E. coli* and on the ability of SacY to replace BglG in antiterminating transcription in *E. coli*, we speculated that sac is regulated similarly to bgl, i.e., that the signal transduction mechanism involves protein phosphorylation. It was previously suggested that SacY activity might be regulated by phosphorylation (17). To examine this hypothesis, we sought to determine whether SacY forms, which differ in their charges, are present in growing *B. subtilis* cells. Such forms can be separated from one another and from other proteins in the cell by the use of 2-D gels. Phosphorylation of BglG in vivo was demonstrated in this way (3). To allow detection of SacY, we expressed it from plasmid pMI3, which harbors sac under control of the inducible Pspac promoter, and labeled the cellular proteins with [35S]methionine. Efficient expression of SacY in *B. subtilis* cells containing pMI3, depending exclusively upon the addition of IPTG to the growth medium, was demonstrated by SDS-PAGE followed by Western blot analysis with anti-SacY antibodies (Fig. 1, compare lanes 1 and 2). We then labeled *B. subtilis* cells metaboli
cally with [35S]methionine in the presence and absence of IPTG and analyzed the labeled proteins on 2-D gels. Two labeled spots, with a molecular size expected for SacY, were detected only in cells treated with IPTG (Fig. 2, compare A and B). These spots were immunoprecipitated by anti-SacY antibodies (Fig. 2A, insert). We could thus conclude that two forms of SacY are present in *B. subtilis* cells.

To test whether the two forms of SacY represent phosphorylated and nonphosphorylated forms of the protein, we treated the 35S-labeled cellular proteins with alkaline phosphatase before gel analysis. This treatment led to the loss of the more acidic form of SacY (compare Fig. 3A, nontreated, to 3B, phosphatase treated), indicating that this indeed is the phosphorylated form (as expected from the charge conferred by a phosphoryl group). This result shows that SacY is phosphory-

**TABLE 1. SacY can antiterminate transcription of a chromosomal bgl-lacZ fusion in *E. coli***

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Plasmid-encoded protein</th>
<th>Phenotype on MacConkey-lactose medium</th>
<th>β-Galactosidase activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMN25</td>
<td>BglG</td>
<td>+</td>
<td>47</td>
</tr>
<tr>
<td>pSL85-XΔ1</td>
<td>SacY</td>
<td>+</td>
<td>39</td>
</tr>
<tr>
<td>pOAACY</td>
<td>SacY</td>
<td>+</td>
<td>51</td>
</tr>
<tr>
<td>pBR322</td>
<td></td>
<td>–</td>
<td>3</td>
</tr>
</tbody>
</table>

* The experiment was carried out with MA152, an *E. coli* strain which is Δbgl and carries a bgl-lacZ transcriptional fusion (35).

* Expression of the bgl-lacZ fusion was partly determined by colony color on MacConkey-lactose plates: +, red colonies; –, white colonies.

* The values, in Miller units, represent the averages of four independent measurements.

**FIG. 1. Induction of sacY expression from the Pspac promoter in *B. subtilis*.** Proteins were extracted from *B. subtilis* cells, containing the sacY gene cloned under Pspac promoter control on plasmid pMI3, which were grown either without (lane 1) or with (lane 2) IPTG. Samples were analyzed by SDS-PAGE, followed by Western blot analysis with anti-SacY antibodies. Molecular masses of protein standards are given in kilodaltons.

**FIG. 2. SacY protein is present in two forms in vivo.** (A) *B. subtilis* cells containing the sacY gene cloned under Pspac promoter control on plasmid pMI3 were induced for SacY production and labeled with [35S]methionine, and analyzed by 2-D gel electrophoresis (isoelectric focusing in one dimension and SDS-PAGE in the second dimension), followed by autoradiography. The insert (top, right) shows fractionation of the same proteins, which were immunoprecipitated with anti-SacY antibodies prior to the 2-D gel analysis. (B) The same cells as described for panel A except that IPTG was not added to the cells. Arrows indicate the positions of the two forms of SacY. Molecular masses of protein standards are given in kilodaltons.

**FIG. 3. SacY is phosphorylated in vivo.** (A) *B. subtilis* cells containing the sacY gene cloned under Pspac promoter control on plasmid pMI3 were induced for SacY production and labeled with [35S]methionine, and proteins were extracted and fractionated as described in the legend for Fig. 2A. (B) The same as described for panel A, except that the extracted proteins were treated with CIP prior to the 2-D gel analysis. Closed arrow indicate nonphosphorylated SacY; open arrows indicate phosphorylated SacY.
induced for SacY production and labeled with \([^{35}S]\)methionine, and proteins were extracted and fractionated as described in the legend for Fig. 2A. The same as described for panel A, except that 30 mM sucrose was added to the growth medium together with the IPTG. Closed arrows indicate nonphosphorylated SacY; open arrows indicate phosphorylated SacY.

In order to determine whether sucrose prevents SacY phosphorylation or can actually lead to dephosphorylation of this protein, similarly to the ability of \(\beta\)-glucosides to lead to BglG dephosphorylation (2, 3), we carried out pulse-chase experiments in which sucrose was added only after SacY had been phosphorylated in the cell. Addition of unlabeled methionine to cells which were pulse-labeled with \([^{35}S]\)methionine did not reduce the relative amount of phosphorylated SacY (compare Fig. 5A and B). However, when sucrose was added to the cells together with the unlabeled methionine, all of the phosphorylated SacY was converted to the nonphosphorylated form together with the unlabeled methionine, all of the phosphorylated form, and we can therefore conclude that the extent of SacY dephosphorylation in vivo is influenced by the presence of sucrose in the growth medium.

In order to determine whether sucrose prevents SacY phosphorylation or can actually lead to dephosphorylation of this protein, we repeated all the controls rather than comparing different experiments.

**Sucrose affects the state of SacY phosphorylation.** SacY antiterminates transcription of the \(sacB\) gene in response to the presence of sucrose in the growth medium. Its \(E.\ coli\) homolog, BglG, antiterminates transcription of the \(bgl\) operon, depending on the presence of \(\beta\)-glucosides in the medium. Therefore, we expected sucrose to influence the state of SacY phosphorylation analogously to the known effect of \(\beta\)-glucosides on the extent of BglG phosphorylation (2, 3). To test this hypothesis, sucrose was added to the growth medium of \(B.\ subtilis\) cells during SacY production at a final concentration of 30 mM (the concentration which leads to SacY-dependent \(sacB\) expression). Cells overproducing SacY in the absence of sucrose served as a control. Whereas the fraction of the phosphorylated form of SacY in the absence of sucrose was approximately 40% (Fig. 4A), this form could hardly be detected when sucrose was added in the medium (Fig. 4B). Thus, in the presence of sucrose, SacY is present mainly in its nonphosphorylated form, and we can therefore conclude that the extent of SacY phosphorylation in vivo is influenced by the presence of sucrose in the growth medium.

To determine whether the fraction of phosphorylated SacY detected in this strain varied between 30 to 60% in different experiments, depending on the conditions of the induction and the length of the pulse-labeling and whether a short chase period was included in the experiment. Therefore, in each of the experiments described below, we repeated all the controls rather than comparing different experiments.

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**The effect of SacX on the state of SacY phosphorylation.** Expression of \(sacB\), which is positively regulated by SacY, is repressed by SacX, a PTS enzyme II-like protein (9). Analogously to the negative regulation of BglG due to its phosphorylation by BglF, an enzyme II of PTS (2, 3), it was suggested that SacX negatively regulates SacY activity by phosphorylating it (17). To determine whether SacX is involved in SacY phosphorylation, we asked whether the extent of SacY phosphorylation correlates with the level of SacX produced in the cell. We therefore analyzed the extent of SacY phosphorylation in cells producing different levels of SacX. To this end, we constructed plasmids pM15 and pM15\(^{\text{ATG}}\), both carrying \(sacX\) and \(sacY\) cloned after Pspac, but whereas the first carries the wild-type \(sacY\) gene, which starts with a TTG codon, the second carries a \(sacX\) allele, which starts with ATG, a more efficient initiation codon than TTG (53). SDS-PAGE analysis of labeled proteins from \(B.\ subtilis\) strains which carry a chromosomal copy of \(sacX\) and contain either pM13 (carrying \(sacY\) alone), pM15, or pM15\(^{\text{ATG}}\) revealed a protein with the molecular size expected for SacX only when SacX was overproduced from the plasmids, as expected (data not shown). It is worth mentioning that the level of overexpressed SacX, even from the more efficiently translated allele, was lower than the level of overexpressed SacY, similar to the case of BglF and BglG (3). Analysis by 2-D gel electrophoresis of SacY produced in the three cell backgrounds, in the absence of sucrose, indicated that the fraction of phosphorylated SacY increased with increased SacX production (Fig. 6). The approximate fraction of SacY present in the phosphorylated form was 30%...
The bgl system in *E. coli*, composed of a membrane-bound sensor, BglF, and a cytoplasmic regulator, BglG, is not a member of the known family of two-component sensory systems (reviewed in references 40, 41, and 49). BglF and BglG have no homology with the sensors and regulators of the two-component systems, respectively. Moreover, it was recently shown that BglG is phosphorylated on a histidine residue, unlike response regulators of the two-component family, which are phosphorylated on an aspartate (6). Thus, *bgl* represents a novel family of systems involved in processing sensory data (reviewed in reference 5). Based on sequence homology and mechanistic similarity, other systems, responding to the presence of various sugars, were suggested to affiliate to this family (see below). Recent findings, showing that the two-component family and histidine phosphorylation are not confined to prokaryotes (14, 33, 39), raise the possibility that eukaryotic systems of the *bgl* family will be found in the future. To elucidate the general features of the mechanism that governs signal transduction in the *bgl* family, it is important to study other members of this family. Does the communication between sensors and regulators of the new family involve reversible phosphorylation, which depends on the presence of the respective sugars, as a general theme?

BglG and BglF have considerable homology with pairs of regulatory proteins, including the *B. subtilis* pairs SacY and SacX (48, 55), SacT and SacP (18, 21), LiT and BglP (29, 31, 45), and LeVr and Lev-PTS (a complex of three proteins) (19, 35), and the *Erwinia chrysanthemi* pair ArbG and ArbF (20).

Characterization of the bond between SacY and the phosphoryl group. Phosphorylation on histidine and aspartate residues (which form phosphoramidates and acyl phosphates, respectively) is widespread among prokaryotes, although it also occurs on other residues (42). BglG is phosphorylated on a histidine (6, 15), while regulators of the two-component family are phosphorylated on an aspartate (see Discussion). To study the nature of the bond between SacY and the phosphoryl group, we tested the stability of P–SacY under conditions which destabilize acyl phosphates and phosphoramidates but not phosphate esters. Only the former two are susceptible to rapid aminolysis at a pH of ~5.5 in the presence of hydroxylamine (12, 26). We therefore incubated an extract of cells overproducing SacY with hydroxylamine in acetate buffer, pH 5.2. This led to the complete disappearance of P~SacY (Fig. 7A). Incubation in acetate buffer (pH 5.2) without hydroxylamine did not affect the ratio between phosphorylated and nonphosphorylated SacY, and the result was as in the control presented in Fig. 7C (data not shown). This result indicates that SacY-phosphate is present either as an N-phosphate or as an acyl phosphate. Because both compounds are known for their relative sensitivity to elevated temperatures (1), we tested the stability of P–SacY to heat by boiling the proteins extracted from cells overproducing SacY. This treatment also resulted in a sharp decrease in the intensity of the phosphorylated form of SacY (Fig. 7B). A new spot migrating between the two forms of SacY, which are routinely observed, appeared after the boiling. The fraction of the protein transferred to this intermediate location did not decrease upon prolonged heating. An explanation for the stability of this new form to heat is suggested in the Discussion.

**FIG. 7. Sensitivity of the bond between SacY and the phosphoryl group to hydroxylamine and heat.** *B. subtilis* cells containing the *sacY* gene cloned in plasmid pMI3 were induced for SacY production and labeled with [35S]methionine. Proteins were extracted and were either subjected to treatment with hydroxylamine as described in Materials and Methods (A) or boiled for 10 min (B). Treated samples and an untreated control (C) were analyzed by 2-D gel electrophoresis, followed by autoradiography. Closed arrows indicate nonphosphorylated SacY; open arrows indicate phosphorylated SacY.

**DISCUSSION**

in the first background (Fig. 6A), 50% in the second (Fig. 6B), and more than 70% in the third (Fig. 6C). This experiment was carried out under conditions that result in a relatively low extent of SacY phosphorylation in the absence of SacX overproduction (short induction period, low level of inducer, relatively short pulse, and no chase). However, a similar dependence of SacY phosphorylation on the level of SacX production was also observed under other experimental conditions. Based on these results, we suggest that the extent of SacY phosphorylation is proportional to the level of SacX in the cell and thus that SacX appears to be involved in SacY phosphorylation.

**Characterization of the bond between SacY and the phosphoryl group.** Phosphorylation on histidine and aspartate residues (which form phosphoramidates and acyl phosphates, respectively) is widespread among prokaryotes, although it also occurs on other residues (42). BglG is phosphorylated on a histidine (6, 15), while regulators of the two-component family are phosphorylated on an aspartate (see Discussion). To study the nature of the bond between SacY and the phosphoryl group, we tested the stability of P–SacY under conditions which destabilize acyl phosphates and phosphoramidates but not phosphate esters. Only the former two are susceptible to rapid aminolysis at a pH of ~5.5 in the presence of hydroxylamine (12, 26). We therefore incubated an extract of cells overproducing SacY with hydroxylamine in acetate buffer, pH 5.2. This led to the complete disappearance of P~SacY (Fig. 7A). Incubation in acetate buffer (pH 5.2) without hydroxylamine did not affect the ratio between phosphorylated and nonphosphorylated SacY, and the result was as in the control presented in Fig. 7C (data not shown). This result indicates that SacY-phosphate is present either as an N-phosphate or as an acyl phosphate. Because both compounds are known for their relative sensitivity to elevated temperatures (1), we tested the stability of P–SacY to heat by boiling the proteins extracted from cells overproducing SacY. This treatment also resulted in a sharp decrease in the intensity of the phosphorylated form of SacY (Fig. 7B). A new spot migrating between the two forms of SacY, which are routinely observed, appeared after the boiling. The fraction of the protein transferred to this intermediate location did not decrease upon prolonged heating. An explanation for the stability of this new form to heat is suggested in the Discussion.

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ratio of less than 1:200 between BglF and BglG was enough to phosphorylate 50 to 60% of the overproduced BglG [3]), the observed phosphorylation of overproduced SacY by SacX was expressed from a chromosomal gene is expected. Our results suggest that SacX is involved in SacY phosphorylation but do not rule out the possibility that the effect of SacX on SacY phosphorylation is indirect. It is hoped that future in vitro studies of SacY phosphorylation will answer the question of whether SacX is the SacY kinase.

Genetic studies have demonstrated that the general PTS proteins, enzyme I and HPr, negatively regulate SacY activity, and it was suggested that they exert this effect through SacX (17). Interestingly, unlike BglG and SacY, the BglG-like proteins SacT and LevR are positively regulated by the general PTS proteins and were shown to be phosphorylated by them in vitro (7, 8, 51). Thus, the general PTS proteins repress the activity of some antiterminators from the bgl family and activate others. The ability of SacY to antiterminate transcription of the bgl operon in E. coli at a level comparable to BglG (Table 1) rules out the possibility that the E. coli general PTS proteins can negatively regulate SacY activity by phosphorylation. Nevertheless, the possibility that the B. subtilis enzyme I and HPr exert a negative effect on SacY activity by directly phosphorylating it, in addition to their indirect effect via SacX, cannot be ruled out.

Another question is whether the phosphorylation of BglG on a histidine residue is a general theme in the bgl family of sensory systems, similarly to the phosphorylation of the response regulators of the two-component systems on an aspartate. The sensitivity of SacY-P to hydroxyamine and heat suggests that it is either a phosphoramidate or an acyl phosphate. Based on the types of amino acids that are known to form these types of bonds in proteins, phosphorylation is suggested to occur either on a histidine or an aspartate. One approach to decide between these possibilities is to determine the stability of SacY-P to acid and base, as was done with BglG-P (6). While phosphoramidates are stable in basic conditions but sensitive to acidic conditions (22), acyl phosphates are labile at either pH extreme (28). We tried to incubate extracts of cells overproducing SacY in HCl and NaOH prior to their analysis, but the 2-D gel technique turned out to be sensitive to such harsh treatments of the analyzed proteins, and the samples precipitated and smeared in a way that made the interpretation of the results difficult. However, the appearance, after boiling, of an additional spot on the 2-D gel, which migrates between the two forms of SacY, provides a hint about the type of amino acid which is phosphorylated in this protein. The most plausible explanation for this phenomenon is the occurrence of a phosphotransfer, i.e., the phosphoryl is transferred to a nearby residue (not necessarily adjacent on the primary sequence) to form a phosphoester which is heat stable. The magnitude of the mobility shift caused by phosphorylation on an aspartate in isoelectric focusing is smaller than that of any other shift which is caused by phosphorylation of other amino acids (13, 16). Therefore, a phosphotransfer from an aspartate to a serine, threonine, or tyrosine should not lead to the appearance of a spot with intermediary migration behavior but, rather, to a shift to the other direction, i.e., to a more acidic position. Phosphotransfer from a histidine to the phosphoester-forming residues will generate the observed shift, both in direction and in magnitude. Thus, this result supports the notion that SacY is phosphorylated on a histidine rather than an aspartate. Theoretically, phosphorylation on a lysine or an arginine, though not discovered yet, should yield the same result and cannot be ruled out. An alternative explanation to this result is phosphorylation of SacY on two histidines or aspartates, combined with a higher sensitivity of one of the two to heat, due to a difference in their immediate surroundings. This explanation is unfavorable because, contrary to what we have observed, prolonged heating in this case should lead to a decrease in the intensity of the intermediate spot.

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