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Requirement of the dephospho-form of enzyme IIA^{Ntr} for derepression of *Escherichia coli* K-12 *ilvBN* expression

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Summary

While the proteins of the phosphoenolpyruvate:carbohydrate phosphotransferase system (carbohydrate PTS) have been shown to regulate numerous targets, little such information is available for the nitrogen-metabolic phosphotransferase system (nitrogen-metabolic PTS). To elucidate the physiological role of the nitrogen-metabolic PTS, we carried out phenotype microarray (PM) analysis with *Escherichia coli* K-12 strain MG1655 deleted for the *ptsP* gene encoding the first enzyme of the nitrogen-metabolic PTS. Together with the PM data, growth studies revealed that a *ptsN* (encoding enzyme IIA^{Ntr}) mutant became extremely sensitive to leucine-containing peptides (LCPs), while both *ptsP* (encoding enzyme I^{Ntr}) and *ptsO* (encoding NPr) mutants were more resistant than wild type. The toxicity of LCPs was found to be due to leucine and the dephospho-form of enzyme IIA^{Ntr} was found to be necessary to neutralize leucine toxicity. Further studies showed that the dephospho-form of enzyme IIA^{Ntr} is required for derepression of the *ilvBN* operon encoding acetohydroxy acid synthase I catalysing the first step common to the biosynthesis of the branched-chain amino acids.

Introduction

Bacteria have the capacity to efficiently adapt to environmental changes by sensing the availability of nutrients. The phosphoenolpyruvate (PEP):carbohydrate phosphotransferease system (carbohydrate PTS) plays an important role in such sensing mechanisms. This system mediates the transport of many sugars across the cytoplasmic membrane by a mechanism that couples translocation to phosphorylation of the substrate (Postma *et al*., 1993). Carbohydrate PTS proteins include the cytoplasmic enzyme I (EI) and HPr, which lack sugar specificity, and membranous enzyme II (EII) complexes, each specific for one or a few sugars. The latter complexes usually consist of three proteins or protein domains that are designated EIIA, EIIB and EIIC. The phosphoryl relay proceeds sequentially from PEP to enzyme I, HPr, EIIA, EIIB, and finally to the incoming sugar that is transported across the membrane and concomitantly phosphorylated by EIIC. The carbohydrate PTS is present in a wide variety of Gram-positive and Gram-negative bacteria, but homologues have not been found in archaea or eukaryotes (Saier, 2001). In addition to its primary functions in sugar transport and phosphorylation, this complex protein system is involved in regulatory processes such as chemoreception (Lux *et al*., 1995), catabolite repression (Postma *et al*., 1993), carbohydrate transport and metabolism (Hurley *et al*., 1993; Postma *et al*., 1993; Seok *et al*., 1997a), carbon storage (Seok *et al*., 1997b; 2001; Koo and Seok, 2001), and regulation of Mlc activity to control the expression level of the carbohydrate PTS and related proteins (Lee *et al*., 2000; Tanaka *et al*., 2000; Nam *et al*., 2001). More recently, we found that EIIA^{Glc} of the carbohydrate PTS also regulates the flux between respiration and fermentation pathways by sensing available sugar species via a phosphorylation state-dependent interaction with the fermentation/respiration switch protein FrsA (Koo *et al*., 2004).

Analysis of the *Escherichia coli* genome has revealed new phosphotransferase-encoding genes (Blattner *et al*., 1997; Saier, 2001). The novel nitrogen-metabolic phosphotransferase system (nitrogen-metabolic PTS) proteins, NPr and EIIA^{Ntr} (paralogues of HPr and EIIA^{Fru}, respectively), are encoded by the *ptsO* and *ptsN* genes, respectively, localized to the *rpoN* operon of *E. coli* (Powell *et al*., 1995; Reizer *et al*., 1996). Enzyme I^{Ntr} (EI^{Ntr}; enzyme I paralogue) encoded by the *ptsP* gene, located distant from the *rpoN* cluster on the chromosome, consists of two domains, an N-terminal domain of 127 amino acids homologous to the N-terminal sensory domain of the

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NifA protein of *Azotobacter vinelandii* (Austin *et al*., 1994) and a C-terminal domain of 578 amino acids homologous to all currently sequenced enzymes I. It was shown that the *E. coli* nitrogen-metabolic PTS shows little cross-reactivity with the carbohydrate PTS and consists of the following phosphoryl transfer chain, PEP→EI^{Ntr}→NPr→EIIA^{Ntr} (Rabus *et al*., 1999).

EIIA^{Ntr} has been implicated in the regulation of the essential GTPase, Era, which appears to function in cell cycle progression and the initiation of cell division in *E. coli* (Powell *et al*., 1995; Britton *et al*., 1998). *E. coli ptsN* deletion mutants, lacking EIIA^{Ntr}, exhibited slower growth in minimal medium containing an organic nitrogen source but not an ammonium salt (Powell *et al*., 1995). EIIA^{Ntr} homologues have been identified in numerous Gram-negative bacteria, and a link between the *ptsN* gene and nitrogen regulation has been suggested for *Rhizobium etli* (Michiels *et al*., 1998), *Pseudomonas aeruginosa* (Jin *et al*., 1994) and *Klebsiella pneumoniae* (Merrick and Coppard, 1989).

Although numerous regulatory activities are associated with the carbohydrate PTS, little is known concerning the function of the nitrogen-metabolic PTS in *E. coli*. In this study, we carried out phenotype microarray (PM) analysis to identify the direct role(s) of the nitrogen-metabolic PTS. Following studies demonstrated the involvement of dephospho-EIIA^{Ntr} in the regulation of leucine toxicity via derepression of the *ilvBN* operon encoding acetohydroxy acid synthase (AHAS) I.

Results

The nitrogen-metabolic PTS regulates sensitivity of E. coli *to leucine-containing peptides (LCPs)*

As it has been well established that the carbohydrate PTS regulates a variety of physiological processes in addition to effecting the catalysis of sugar uptake, we thought that the nitrogen-metabolic PTS might also have multiple roles in regulating physiological activities in *E. coli*. This perspective stimulated us to perform PM experiments. The PM technique involves the deduction of cellular phenotypes using cell respiration as the reporter system and allows measurement of cell growth to examine nearly 2000 cellular phenotypes in a sensitive, highly controlled, reproducible format (Bochner *et al*., 2001; Zhou *et al*., 2003; Koo *et al*., 2004).

It should be noted that the *ptsN* strain, harbouring a deletion of the gene encoding EIIA^{Ntr}, could not be used for PM tests because, as reported previously (Powell *et al*., 1995), it hardly grew in the presence of succinate or glycerol as the sole carbon source; these are generally used as carbon sources in PM experiments for testing nitrogen, phosphorus and sulphur sources. This growth defect of the *ptsN* mutant in a defined medium makes it difficult to compare its phenotype with that of wild-type cells by employing PM analyses. PM tests were therefore performed on CR101 (*ptsP::cat*), harbouring a deletion of the gene encoding EI^{Ntr}, in comparison with the control strain MG1655 (data not shown). As we replaced the *ptsP* gene with the *cat* gene, the mutant was more resistant than wild type to chloramphenicol, as expected. The most prominent difference, except for the antibiotic resistance, between the *ptsP* mutant and wild type was shown in tests for growth in the presence of different nitrogen sources. Out of 10 nitrogen sources showing more than 60% difference in the growth of the mutant compared with wild type, seven corresponded to LCPs. The results indicated that CR101 showed better growth than its parental strain MG1655 in medium with LCPs, such as Ala-Leu, Arg-Leu, Leu-Arg, Trp-Leu, Leu-Pro or Gly-Leu, as sole nitrogen source (data not shown). It should be noted that CR101 also grew faster than MG1655 in medium containing the Lys-Ser dipeptide as sole nitrogen source (data not shown).

To confirm and extend the PM data, we compared the growth kinetics of the *ptsP* mutant with wild type in synthetic minimal medium containing glucose as the carbon source supplemented with 0.5 mM or 5 mM of the dipeptide Ala-Leu in the presence or absence of ammonium sulphate as the nitrogen source. The addition of Ala-Leu to the culture of wild-type MG1655 increased the lag period by more than 10 h regardless of the presence of ammonium sulphate in the medium (Fig. 1; data not shown for the growth in the absence of ammonium sulphate), as expected from a previous report (Tavori *et al*., 1981). The *ptsP* mutant (CR101) exhibited a phenotype of significantly faster growth than its parental strain MG1655 in the presence of the dipeptide, confirming the PM data.

In a previous report, it was found that the phosphoryl group transfer chain of the nitrogen-metabolic PTS (PEP→EI^{Ntr}→NPr→EIIA^{Ntr}) is distinct from that of the carbohydrate PTS (PEP→EI→HPr→EIIA^{Carbohydrate}) and it was proposed that EI^{Ntr} may function exclusively in regulation, possibly controlling the activities of NPr and EIIA^{Ntr} encoded within the *rpoN* operon (Rabus *et al*., 1999). To determine whether the phenotype of faster growth of the *ptsP* mutant in the medium supplemented with the LCPs is through controlling the activities of one of its downstream proteins in the phosphoryl transfer cascade, two mutants, CR201, deleted for *ptsO* (encoding NPr) and CR301, deleted for *ptsN* (encoding EIIA^{Ntr}) were constructed. As shown in Fig. 1, the *ptsO* mutant grew even faster than the *ptsP* mutant, especially in the presence of the higher concentration of Ala-Leu in the medium, while the *ptsN* mutant hardly grew regardless of the dipeptide concentration used in this experiment. It should be noted

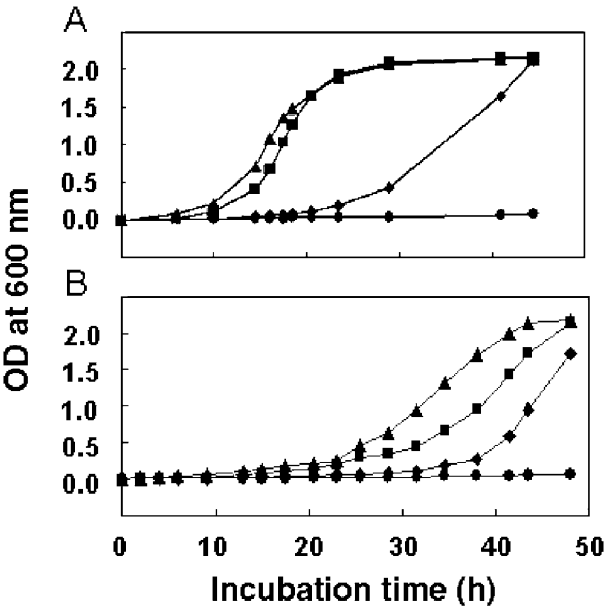

Fig. 1. Effect of Ala-Leu on growth of the nitrogen-metabolic PTS mutants. Cells grown in LB overnight were washed with M9 medium, inoculated in M9 medium containing 0.5% glucose supplemented with 0.5 mM (A) or 5 mM (B) Ala-Leu, and growth was recorded by measuring the optical density at 600 nm: diamonds, MG1655; squares, CR101; triangles, CR201; and circles, CR301.

that the addition of ammonium sulphate as nitrogen source did not change the inhibitory effect of Ala-Leu on growth of the three mutants and wild-type MG1655 (data not shown) and that the apparent lag phase and growth inhibition could be observed when cells pregrown in either Luria–Bertani (LB) (Fig. 1) or M9 (data not shown) were transferred into M9 medium containing Ala-Leu. The growth rate of the *ptsO* mutant CR201 in the M9 medium containing 0.5 mM Ala-Leu dipeptide was comparable to that of MG1655 in the medium without addition of the dipeptide (data not shown). These results imply that the nitrogen-metabolic PTS influences the sensitivity of *E. coli* to LCPs and the *ptsN* gene product is necessary for growth of cells in medium containing LCPs.

Toxicity of LCPs is due to leucine and the dephospho-form of EIIA^{Ntr} is necessary to neutralize leucine toxicity

It was previously reported that a variety of LCPs inhibited the growth of a prototrophic strain of *E. coli* K-12 in minimal medium by increasing the lag period by up to 10 h; after the lag period, the rate of growth was similar to the control culture grown in the absence of LCPs (Tavori *et al*., 1981). It was shown that the concentration of leucine inside bacteria treated with LCPs was higher than that in the leucine-treated or the control cultures and proposed that the growth inhibition by LCPs was due to accumulation of abnormally high concentrations of leucine in the cell, although the mechanism by which LCPs led to high intracellular leucine has not yet been clarified. It should be noted that the *ptsN* mutant was about 10 times more sensitive to LCPs than to leucine (compare data in Fig. 1 with that of Fig. 2 and Table 1). Taken together with these observations, our data suggested that the *ptsN* gene product, EIIA^{Ntr}, might neutralize leucine toxicity. Accordingly, we checked whether the complete cessation of growth of the *ptsN* mutant in medium supplemented with LCPs was due to leucine or other amino acids found in the peptides. Out of 20 amino acids we tested, only leucine, isoleucine and serine, showed a detrimental effect on the growth of the *ptsN* mutant, while their effects on the MG1655 growth were negligible (Table 1). These amino acids did not show any inhibitory effect on the *ptsP* and *ptsO* mutants (data not shown).

The regulatory functions of the carbohydrate PTS generally depend on the phosphorylation state of the involved protein; the sequential phosphoryl transfer of the nitrogen-metabolic PTS occurs as follows: PEP→EI^{Ntr}→NPr→EIIA^{Ntr}. The faster growth of the *ptsP* and *ptsO* mutants than wild type and the complete loss of growth of the *ptsN*

Table 1. Effect of amino acids on growth of the *ptsN* mutant in minimal medium.

Amino acid added[a]	MG1655	CR301	CR301/pCR3	Amino acid added	MG1655	CR301	CR301/pCR3
None	+++++[b]	++++	+++++	Met	+++++	++++	+++++
Ala	+++++	+++++	+++++	Asn	+++++	++++	+++++
Cys	++	++	++	Pro	+++++	+++++	+++++
Asp	+++++	++++	+++++	Gln	+++++	+++++	+++++
Glu	+++++	++++	+++++	Arg	+++++	++++	+++++
Phe	+++++	++++	+++++	Ser	++++	–	++
Gly	+++++	++++	+++++	Thr	++++	++++	++++
His	+++++	++++	+++++	Val	–	–	–
Ile	++++	+	++	Trp	+++++	++++	+++++
Lys	++++	++++	++	Tyr	+++++	+++++	+++++
Leu	++++	–	+++				

a. M9 minimal medium containing 0.5% glucose was supplemented with the indicated amino acid to a final concentration of 5 mM.
b. Growth rates during the first 24 h at 37°C after inoculation were compared: –, no growth; +, very slow; ++, slow; +++, medium; ++++, faster than medium; +++++, fastest.

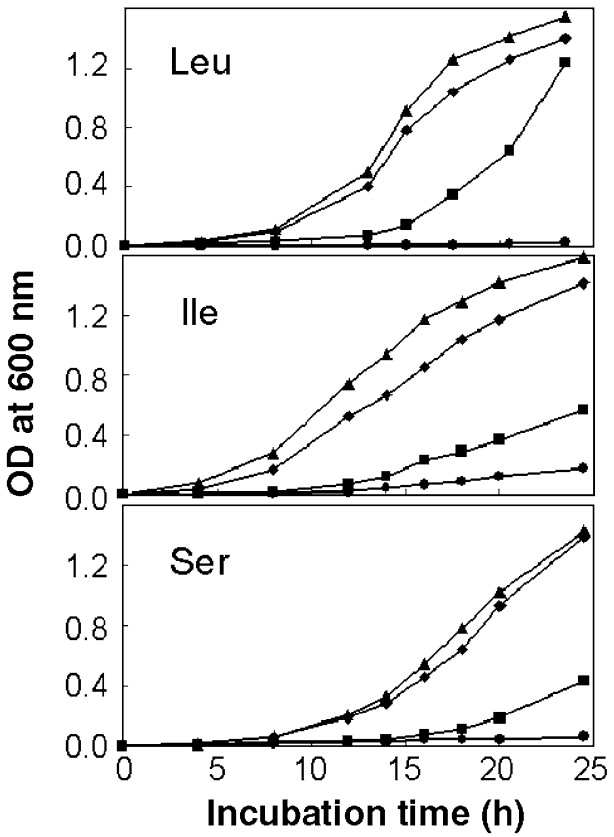

Fig. 2. Effect of the dephospho-form of enzyme IIA^{Ntr} on sensitivity of the *ptsN* mutant to leucine, isoleucine and serine. Growth of CR301 strains transformed with pRE1 (circles), pCR3 (squares) and pCR3(H73A) (triangles) was compared with that of MG1655 harbouring pRE1 (diamonds) in M9 medium containing 0.5% glucose in the presence of the indicated amino acid (5 mM) at 37°C.

mutant imply that the presence of the dephospho-form, but not the absence of the phospho-form, of EIIA^{Ntr} might be necessary for the growth of *E. coli* K-12 cells in medium supplemented with leucine or the LCPs. To confirm this idea, we constructed a pRE1-based plasmid pCR3(H73A) expressing a mutant form of EIIA^{Ntr} in which the phosphorylatable His73 residue was mutated to Ala. As indicated in Table 1, CR301 transformed with pCR3 expressing wild-type EIIA^{Ntr} recovered resistance to isoleucine, serine and leucine, although growth was less efficient than wild type (Fig. 2). Intriguingly, CR301 harbouring pCR3(H73A) expressing the unphosphorylatable form of EIIA^{Ntr} showed enhanced resistance to these amino acids, even more than the wild type. From these results, it became clear that the presence of the dephospho-form of EIIA^{Ntr}, but not the absence of its phospho-form, is necessary to suppress leucine toxicity.

The dephospho-form of EIIA^{Ntr} is required for derepression of AHAS

It seems clear that the inhibition of growth by LCPs is due to the generation of cellular leucine. It was previously reported that AHAS, the first common enzyme in the biosynthetic pathway of the three branched-chain amino acids (see Fig. 3 for the biosynthetic pathway of the branched-chain amino acids), is a target for LCP toxicity in *E. coli* K-12 (Gollop *et al*., 1982). It was shown that AHAS from a mutant resistant to LCPs was also insensitive to inhibition by leucine, which was previously shown to inhibit growth of *E. coli* K-12 by inhibiting AHAS activity and creating a state of isoleucine pseudoauxotrophy (Quay *et al*., 1977; Gollop *et al*., 1982). Wild-type *E. coli*

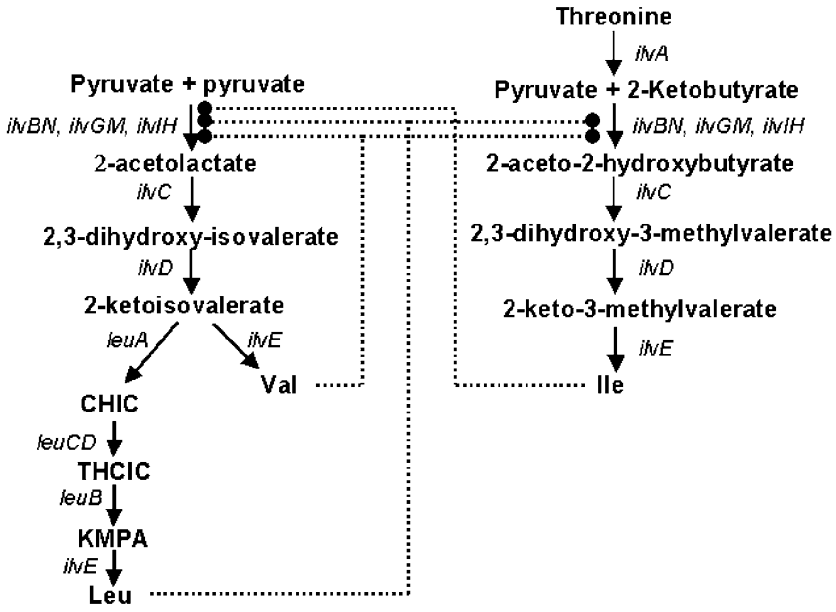

Fig. 3. Biosynthetic pathway of the branched-chain amino acids. In *E. coli*, the first step common to the biosynthesis of the three branched-chain amino acids is catalysed by three isozymes, AHAS I, II and III encoded by *ilvBN*, *ilvGM* and *ilvIH* respectively. In *E. coli* K-12 strains such as MG1655, however, the *ilvG* gene, encoding AHAS II that is insensitive to feedback inhibition by valine, is not expressed due to a frameshift mutation. Dashed lines with filled circles at the ends indicate feedback inhibition showing that one end product inhibits biosynthesis of itself as well as the other end product of the common biosynthetic pathway and induces starvation for the other amino acid. CHIC, 3-carboxy-3-hydroxy-isocaproate; THCIC, 2-D-*threo*-hydroxy-3-carboxy-isocaproate; KMPA, 2-keto-4-methyl-pentanoate.

K-12, such as MG1655, was known to be extremely sensitive to valine because the *ilvG* gene, encoding the AHAS II that is insensitive to feedback inhibition by valine, is not expressed due to a frameshift mutation and exogenous valine inhibits the residual AHAS I and III activities, resulting in a starvation for the other end product of the common biosynthetic pathway, isoleucine, and a state of isoleucine pseudoauxotrophy (Lawther *et al*., 1981). Thus, it was not surprising that both wild type and the *ptsN* mutant could not grow in the presence of valine (Table 1). It is noteworthy that inhibition of growth by leucine or valine could be relieved by addition of isoleucine to the medium (Lawther *et al*., 1981; Gollop *et al*., 1982). Serine toxicity in *E. coli* K-12 was also reported to correlate with the derepressability of *ilv* genes encoding enzymes for the biosynthesis of the branched-chain amino acids including AHAS (Uzan and Danchin, 1978). Taken together with the previous reports, our results suggest that the complete growth inhibition of the *ptsN* mutant by leucine or LCPs might be related to the level of intracellular AHAS activity.

If the complete growth inhibition of the *ptsN* mutant by leucine, serine, isoleucine, or valine is related to the level of intracellular AHAS activity, these amino acids, when added together to the medium, should not show a cumulative effect on growth of the *ptsN* mutant. Instead, the growth inhibitory effect of one amino acid would be relieved by addition of the other amino acid to the medium. Therefore, we checked the effect of the addition of isoleucine, serine, and/or valine on the leucine sensitivity of CR301 (the *ptsN* deletion). The toxic effect of leucine on the growth of the *ptsN* mutant was considerably relieved by addition of isoleucine. Furthermore, the addition of valine and/or serine did not inhibit growth of the mutant in the presence of leucine and isoleucine (Fig. 4). These data strongly suggest that complete growth inhibition of the mutant by leucine or LCPs is related to AHAS activity.

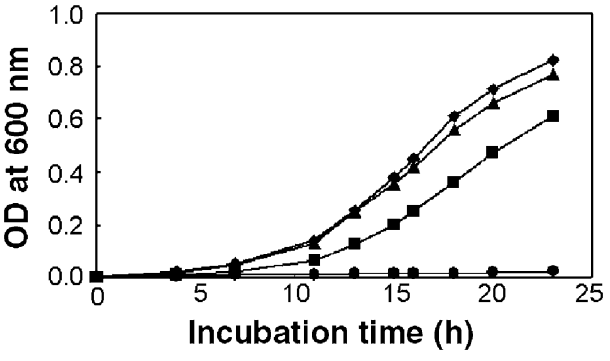

Fig. 4. Effect of isoleucine, valine and serine on the leucine sensitivity of the *ptsN* mutant. Strain CR301 was grown in LB, then harvested, washed and resuspended in M9 minimal medium containing 0.5% glucose and amino acids at 5 mM each as follows: Circles, leucine; squares, leucine + isoleucine; triangles, leucine + isoleucine + valine; and diamonds, leucine + isoleucine + valine + serine.

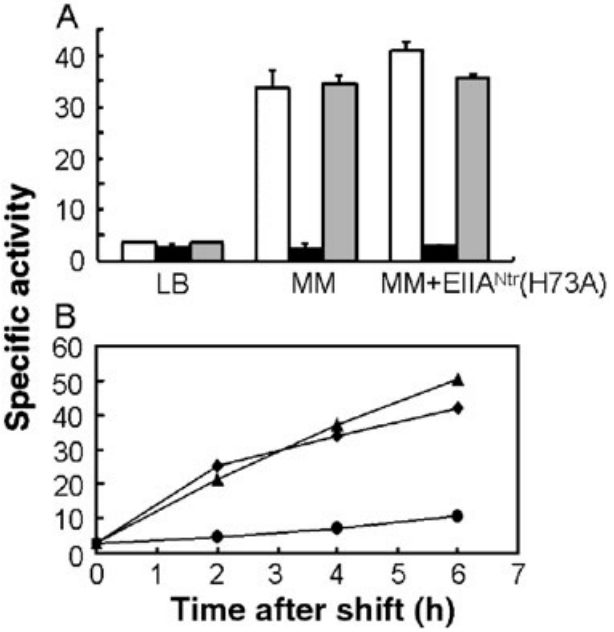

Fig. 5. Comparison of AHAS activities between the *ptsN* mutant and wild type in different growth media.
A. Cells of strains MG1655 (white bar), CR301 (black bar) and CR301 harbouring pCR3(H73A) (grey bar) growing in LB were harvested, washed and resuspended in LB medium (LB) or M9 minimal medium containing 0.5% glucose (MM). Afterward, cells were allowed to grow to mid-logarithmic phase and the specific activity of AHAS was measured as described in *Experimental procedures*. To check the direct effect of the dephospho-form of EIIA^{Ntr} on the AHAS activity, purified EIIA^{Ntr} (3 μg) was also added to the reaction mixture for enzyme activity assay of the crude extract prepared from cells grown in MM [MM + EIIA^{Ntr}(H73A)].
B. Cells growing in LB were harvested, washed and resuspended in M9 minimal medium containing 0.5% lactose and 17 non-branched amino acids (each at a concentration of 0.1 mM). Samples were collected by centrifugation at the indicated times after the nutritional downshift to check the specific activity of AHAS: Diamonds, MG1655; circles, CR301; and triangles, CR301/pCR3(H73A). Specific activity is defined as μmoles acetoin formed per h per mg protein utilizing an acetoin standard curve.

To determine whether the dephospho-form of EIIA^{Ntr} regulates AHAS activity or its expression, we checked the total intracellular activity of AHAS as described in *Experimental procedures.* Because it was previously shown that AHAS expression in *E. coli* is repressed in the presence of leucine (Freundlich, 1977), it might be assumed that AHAS expression would be repressed in a complex medium but derepressed in a minimal medium devoid of amino acids. As predicted, the specific activity of AHAS was very low in wild-type MG1655 cells grown in LB, while it was highly derepressed in cells growing in M9 minimal medium (Fig. 5). However, AHAS activity in the *ptsN* mutant grown in minimal medium was as low as that in cells grown in LB, indicating that derepression of AHAS expression did not occur in the *ptsN* mutant. The cell crude extract of strain CR301 harbouring pCR3(H73A) showed comparable AHAS activity to that prepared from

wild-type cells, regardless of the growth medium (Fig. 5A). It should be noted that episomal expression of EIIA^{Ntr}(H73A) did not result in derepression of AHAS in cells growing in LB medium. Furthermore, the addition of the dephospho-form of EIIA^{Ntr} to the reaction mixture did not affect AHAS activity *in vitro*, strongly suggesting that the presence of EIIA^{Ntr} is required for derepression of the expression of AHAS in the cell and not for an effect on AHAS enzyme activity. To check whether non-branched amino acids can affect derepression of AHAS and whether the activity of β-galactosidase expressed from the chromosomal *lac* operon can be affected by the deletion of *ptsN* as a control, cells growing in LB were harvested, washed and resuspended in M9 minimal medium containing 0.5% lactose and 17 non-branched amino acids. Subsequently, the specific activities of AHAS and β-galactosidase were measured from samples collected at the indicated times after the nutritional downshift. In cells of wild type and CR301 harbouring pCR3(H73A), derepression of AHAS occurred independently of the addition of 17 non-branched amino acids, as expected, while that in the *ptsN* mutant CR301 was completely abolished (Fig. 5B). The activity of β-galactosidase was independent of the *ptsN* mutation (data not shown). These data support the conclusion that the dephospho-form of EIIA^{Ntr} is required for the derepression of AHAS in *E. coli* cells growing in a medium depleted of the branched-chain amino acids.

In *E. coli*, genes in at least three operons code for distinct AHASs: *ilvBN* (AHAS I), *ilvGM* (AHAS II) and *ilvIH* (AHAS III) (Fig. 3; for review, see Umbarger, 1996). In *E. coli* K-12 strains, however, *ilvG* is cryptic due to a frameshift mutation, and only AHAS I and III are actively expressed. AHAS I was known to be responsible for the majority of AHAS activity (Gollop *et al*., 1983). To determine which AHAS requires the dephospho-form of EIIA^{Ntr} for derepression, mRNA levels of the *ilvBN* and *ilvIH* operons was measured by reverse transcription polymerase chain reaction (RT-PCR) in MG1655, CR301 and CR301 harbouring pCR3(H73A) grown in defined medium (Fig. 6A). The transcript level of the *ilvBN* operon was much higher than that of *ilvIH* in wild-type cells, as expected. In the *ptsN* mutant, however, the mRNA level of the *ilvBN* operon was much lower than in wild type, while the expression level of *ilvIH* was hardly affected by the *ptsN* mutation. The leucine-responsive regulatory protein Lrp was known to control the expression of a large number of genes, including the *ilvIH* operon, but not *ilvBN* in *E. coli* (Jafri *et al*., 2002). We found the expression level of *lrp* to be independent of the *ptsN* gene product (Fig. 6A). To confirm the effect of the *ptsN* deletion on the expression of the two operons, we constructed *lacZ* operon fusion strains. As shown in Fig. 6B, the *ptsN* deletion caused a significant decrease in β-galactosidase activity in the strain harbouring the *ilvB–lacZ* construct. In contrast, the effect of *ptsN* deletion on β-galactosidase activity in the strain harbouring *ilvI–lacZ* construct was negligible. The obvious conclusion is that the dephospho-form of EIIA^{Ntr} is required for derepression of the *ilvBN* operon.

Discussion

Earlier experiments indicated that leucine could greatly lengthen the growth lag when *E. coli* K-12 cells were transferred from rich to minimal medium. In addition, it was demonstrated that a mutation leading to derepression of AHAS, catalysing the first step common to the biosynthesis of the branched-chain amino acids, could abolish leucine sensitivity (Rogerson and Freundlich, 1970). In *E. coli* K-12 strains, the *ilvBN* operon encoding AHAS I and the *ilvIH* operon encoding AHAS III are actively expressed (Fig. 3) (Umbarger, 1996). It is a normal occurrence that cells make more proteins necessary to synthesize amino acids when they encounter an environment depleted of those amino acids. The *ilvBN* operon is repressed in cells grown in media containing excess branched-chain amino acids and derepressed when leucine and valine are in short supply. This regulation in response to the supply of branched-chain amino acids is mediated by transcription attenuation (Friden *et al*., 1982). Expression of the *ilvIH* operon is also repressed in cells growing in a medium containing leucine, through the leucine-responsive regulatory protein Lrp (Platko *et al*., 1990). Lrp is modulated by leucine, but not valine or isoleucine, which interferes with DNA binding activity of the protein. Thus, Lrp has been considered to be responsible for greatly increased sensitivity toward growth inhibition by leucine, valine and serine, when it was mutated (Quay *et al*., 1977).

In this study, the results in Fig. 1 showing that the *ptsN* mutant became extremely sensitive to leucine and LCPs, while *ptsP* and *ptsO* mutants were more resistant than wild type, provided us with the clue that the dephospho-form of EIIA^{Ntr} is involved in leucine sensitivity and in derepression of AHAS. Because the phosphoryl group of PEP is sequentially transferred from EI^{Ntr} to EIIA^{Ntr} via NPr, EIIA^{Ntr} will always exist in its dephospho-form in *ptsP* and *ptsO* mutants. If there existed another phospho-accepting protein from EIIA^{Ntr} and it were responsible for the leucine sensitivity and derepression of AHAS, the phenotype of the *ptsN* mutant would be the same as that of the *ptsP* and *ptsO* mutants. The completely different phenotype of the *ptsN* mutant compared with that of the *ptsP* and *ptsO* mutants, however, excluded the possibility for the involvement of another protein downstream of EIIA^{Ntr} and led us to the conclusion that the dephospho-form of EIIA^{Ntr} makes cells more resistant to leucine and LCPs in *ptsP* and *ptsO* mutants. Further experiments using the *ptsN*

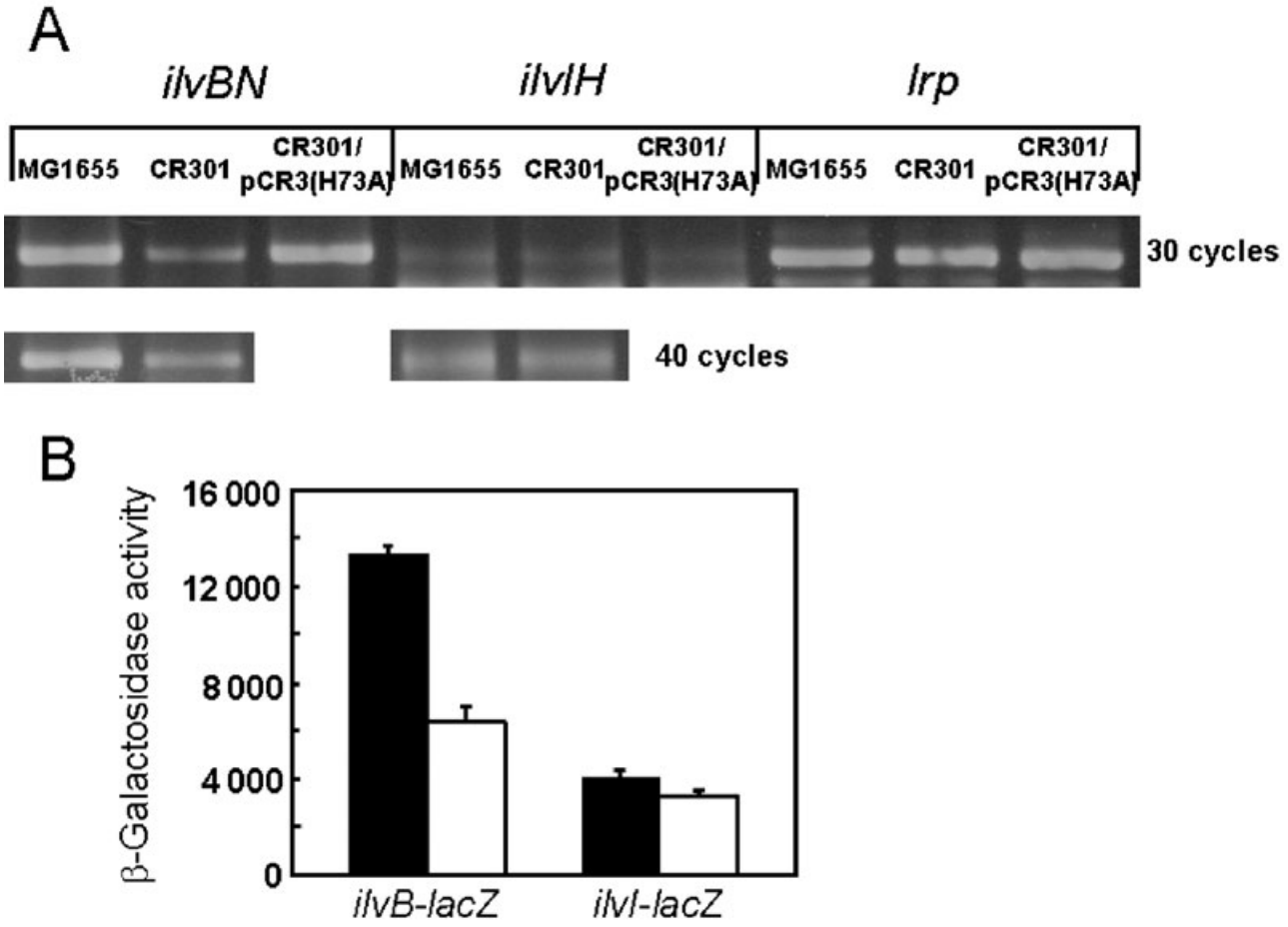

Fig. 6. Effect of EIIA^{Ntr} on expression of *ilvBN* and *ilvIH* operons.
A. Cells were grown in M9 medium containing 0.5% glucose and harvested at exponential growth phase. Total mRNA prepared as described in *Experimental procedures* was used for RT-PCR to measure the expression level of *ilvBN* and *ilvIH* operons and the *lrp* gene after amplification for 30 or 40 cycles as indicated. The products were separated on 1.5% agarose gels, stained with ethidium bromide, and visualized with UV transillumination.
B. β-Galactosidase activity was measured from CR401 and CR501 cells grown to mid-logarithmic phase. Shaded bars correspond to the control strains CR400 (*ilvB–lacZ*) and CR500(*ilvI–lacZ*); open bars correspond to the *ptsN* deletions CR401(*ilvB–lacZ*) and CR501(*ilvI–lacZ*). The values are the means of three independent cultures measured in Miller units (Miller, 1972).

mutant harbouring pCR3(H73A) expressing the unphosphorylatable form of EIIA^{Ntr} confirmed this assumption (Fig. 2).

Studies on the level of mRNAs and β-galactosidase activities in operon fusion strains revealed that the dephospho-form of EIIA^{Ntr} is required for derepression of *ilvBN* encoding AHAS I, responsible for the majority of AHAS activity (Fig. 6). Together with the results from RT-PCR and β-galactosidase activity analyses shown in Fig. 6, the finding that EIIA^{Ntr} did not interact with purified Lrp regardless of the presence of leucine (data not shown) excluded the possibility for the involvement of EIIA^{Ntr} in the regulation of Lrp activity and *ilvIH* derepression. The result showing that *ilvB–lacZ* expression was decreased only twofold while the RT-PCR of *ilvB* transcript and AHAS activity experiments showed much greater decrease in the *ptsN* mutant compared with wild type may suggest a post-transcriptional control of *ilvBN* expression by EIIA^{Ntr}.

Because *ptsN* and *ptsO* genes are localized to the *rpoN* operon, it could be assumed that these gene products might be involved in nitrogen metabolism. It was reported that *ptsN* deletion mutants of *E. coli* exhibited slower growth in minimal medium containing organic nitrogen sources, such as glutamine, aspartate, or alanine, but the presence of an ammonium salt suppressed the growth defect (Powell *et al*., 1995). In this study, we found a new role of the *ptsN* gene product in nitrogen metabolism. Specifically, the dephospho-form of EIIA^{Ntr} is required for the derepression of AHAS I in *E. coli* cells growing in medium depleted of the branched-chain amino acids, and thus, an excess of leucine, isoleucine, or serine is detrimental to growth of the *ptsN* mutant, even in the presence

of an ammonium salt. Without derepression of AHAS I, AHAS III might be responsible for the majority of AHAS activity. As valine, isoleucine and leucine were known to inhibit AHAS III activity (Umbarger, 1996), the presence of one amino acid in the medium will inhibit synthesis of the other amino acid (Fig. 3). This can explain the reason for the growth defect of the *ptsN* mutant in the presence of isoleucine or leucine. Although serine toxicity in *E. coli* K-12 was reported to correlate with the derepressibility of AHAS (Uzan and Danchin, 1978), the mechanism of this effect remains to be clarified.

While we demonstrated that the dephospho-form of EIIA^{Ntr} is required for the derepression of AHAS I, the precise mechanism has not been elucidated. We showed that the dephospho-form of EIIA^{Ntr} is involved in the regulation of AHAS expression, but not in the regulation of AHAS activity (Fig. 5). Derepression of AHAS I in the presence of the dephospho-form of EIIA^{Ntr} probably requires another unknown factor in that expression of EIIA^{Ntr}(H73A) did not result in derepression of AHAS activity in cells growing in LB medium.

Previously, we and other researchers described the role of the major glucose transporter EIICB^{Glc} of the carbohydrate PTS in the direct regulation of Mlc activity (Lee *et al.*, 2000; Tanaka *et al.*, 2000; Nam *et al.*, 2001). The dephospho-form of EIICB^{Glc}, resulting from the presence of glucose, can relieve the repression of the Mlc regulon by sequestering Mlc through a direct protein-protein interaction to induce expression of the Mlc regulon including the carbohydrate PTS proteins. Overexpression of EIICB^{Glc} increased PTS expression even without glucose. In a similar fashion, if the dephospho-form of EIIA^{Ntr} directly mediates derepression of AHAS I, its expression should induce expression of AHAS I even in cells grown in rich medium. The result in Fig. 5 suggests that the effect of the dephospho-form of EIIA^{Ntr} on derepression of AHAS I might be through an uncharacterized transcription factor acting on the *ilvBN* promoter. In summary, we postulate from results in this study that, when cells are transferred from medium containing excess branched-chain amino acids to minimal medium, proteins of the nitrogen-metabolic PTS are dephosphorylated and the dephospho-form of EIIA^{Ntr} sequesters an uncharacterized transcription factor to derepress expression of *ilvBN* necessary to synthesize the branched-chain amino acids. Up to this point, our initial searches for a protein that interacts with EIIA^{Ntr} have been unsuccessful. Additional studies are required to elucidate the connection between the availability of the branched-chain amino acids and the phosphorylation state of the nitrogen-metabolic PTS and that between the dephospho-form of EIIA^{Ntr} and AHAS I expression.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. LB was used as the complex culture medium for the routine growth of bacteria unless indicated otherwise. For synthetic minimal medium, M9 medium containing 0.5% glucose, supplemented with the indicated amounts of amino acids if necessary, was used. For the growth of GI698 strains, M9 salts-based rich medium was used as described previ-

Table 2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Source or reference
Strains		
DY330	W3110 Δ*lacU169 gal490 λCI857 Δ(cro-bioA)*	Yu *et al.* (2000)
GI698	F$^-$ λ$^-$ *lacI*^{q} *lacPL8 ampC*::P$_{trp}$ cI	LaVallie *et al.* (1993)
JM101	*supE thi-1* Δ(*lac-proAB*) *F* [*traD26 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	Miller (1972)
MG1655	Wild type *E. coli* K-12	Blattner *et al.* (1997)
CR100	DY330 *ptsP::cat*	This study
CR101	MG1655 *ptsP::cat*	This study
CR200	DY330 *ptsO::*Tet^R	This study
CR201	MG1655 *ptsO::*Tet^R	This study
CR300	DY330 *ptsN::*Tet^R	This study
CR301	MG1655 *ptsN::*Tet^R	This study
CR400	JM101 λ[Φ(*ilvB′–′lacZ*)]	This study
CR401	CR400 *ptsN::*Tet^R	This study
CR500	JM101 λ[Φ(*ilvI′–′lacZ*)]	This study
CR501	CR500 *ptsN::*Tet^R	This study
YJ2001	MC4100 *arcA::cat*	Jeong *et al.* (2004)
YJ2004	DY330 *mlc::*Tet^R	Jeong *et al.* (2004)
Plasmids		
pRE1	Expression vector under control of λP$_L$ promoter, Amp^r	Reddy *et al.* (1989)
pRS415	*lacZ lacY*$^+$ *lacA*$^+$, Amp^r	Simons *et al.* (1987)
pCR3	pRE1-based expression vector for EIIA^{Ntr}	This study
pCR3(H73A)	pRE1-based expression vector for EIIA^{Ntr}(H73A)	This study
pCR4	pRS415[Φ(*ilvB′-′lacZ*)], Amp^r	This study
pCR5	pRS415[Φ(*ilvI′-′lacZ*)], Amp^r	This study

ously (LaVallie *et al*., 1993; Seok *et al*., 1997b; Jeong *et al*., 2004). DY330, GI698 and their derivatives were grown at 30°C, while the other strains listed in Table 2 were grown at 37°C.

The *ptsP* deletion mutant was constructed using *E. coli* DY330 as described previously (Yu *et al*., 2000). The *ptsP* gene (from the start codon to the stop codon) was replaced by the chloramphenicol acetyltransferase gene (*cat*). The *cat* gene was amplified by PCR from the YJ2001 strain (Jeong *et al*., 2004) with the following primers: forward primer, 5′-CCG CGA ATC AGC CCG CCC ATG CCG CGA CGC TCC ATA AAG GCT GCA ACC ATA CGC CCC GCC CTG CCA CT-3′ and reverse primer, 5′-CAC AAA ACG CAT CTG CTT ATC GAC GTA AAA GAG GTT AAG TCA CGC CAA TAT GGA GAA AAA AAT CAC TGG-3′. The PCR product was electroporated into *E. coli* DY330 to make the strain CR100. MG1655 Δ*ptsP* (CR101) was constructed by P1 transduction of the Cm^r region of CR100. Strains CR200 (DY330 *ptsO*::Tet^R) and CR201 (MG1655 *ptsO*::Tet^R) were prepared in a similar way using primers 5′-CAG TCA CGC CAT CGT ACG CTG GAA AAA CGT AAA CCA TGA CCG TCA AGC AAT TAA GAC CCA CTT TCA CAT T-3′ and 5′-CTT ATT GTC GGG GGG AGT TTG AAG GGA GTT GTA TGT CAA AGT GAT GAA GAC TAA GCA CTT GTC TCC TGTT-3′ to amplify the Tet^R region from the chromosomal DNA of YJ2004 (Jeong *et al*., 2004) and replace the coding region of *ptsO* with that of the Tet^R gene. To prepare the *ptsN* deletion strains, CR300 (DY330 *ptsN*::Tet^R) and CR301 (MG1655 *ptsN*::Tet^R), we used primers 5′-TGC TCC GAG CCT GTT CCA CTG TTT GAG TGG GCA GGT TCT TAG GTG AAA TTG GAA AAA GGT TAT GCT GCTT-3′ and 5′-ACC ATG TAC TGT TTC TCC TCA CAA CGT CTA AAA GAG ACA TTA CCG AAT AAA TCA TTT GGT GAC GAA ATAA-3′. The Tet^R region was moved from DY330 strains to MG1655 strains by P1 transduction. Deletion of the three genes was confirmed by PCR.

Purification of EIIA^{Ntr} and EIIA^{Ntr}(H73A)

To construct pCR3, the vector for EIIA^{Ntr} expression, primers possessing the synthetic restriction enzyme sites NdeI, located 3 bp upstream from the ATG start codon (in boldface type) (5′-GGTTCTTAGGTGAACAT**ATG**ACAAATAATG-3′), and SalI, located 41 bp downstream from the TAA stop codon (5′-GTACATGGATCCGATGATCGTCAGCGGACG-3′) (restriction sites underlined) of the *ptsN* gene were used to amplify, by PCR, the *ptsN* gene from MG1655 genomic DNA. After digestion, the NdeI-SalI fragment was inserted into the corresponding sites of pRE1 (Reddy *et al*., 1989) to make pCR3. The expression vector pCR3(H73A) for overproduction of EIIA^{Ntr}(H73A) was generated in a similar manner using an additional mutagenic primer pair covering the region coding for His73: forward primer, 5′-ATT GCC ATT CCG GCT GGC AAA CTC GAG GAA GAT ACT CTG-3′; reverse primer, 5′-CAG AGT ATC TTC CTC GAG TTT GCC AGC CGG AAT GGC AAT-3′ (changed bases underlined). *E. coli* GI698 harbouring pCR3(H73A) was grown and expression of EIIA^{Ntr}(H73A) was induced as described previously for overproduction of other proteins (LaVallie *et al*., 1993; Seok *et al*., 1996; Koo *et al*., 2004). The cell pellet obtained from 500 ml of culture containing overexpressed EIIA^{Ntr}(H73A) was resuspended in buffer A (10 mM Tris·HCl, pH 7.5, containing 50 mM NaCl) and then passed two times through a French pressure cell at 10 000 p.s.i. The lysate was cleared of cell debris by centrifugation at 100 000 *g* for 90 min. The soluble fraction was chromatographed through a DEAE-Sepharose (Sigma) column (2.5 × 10 cm) using a gradient of 50–500 mM NaCl (200 ml). The fractions containing substantial amounts of EIIA^{Ntr}(H73A) were pooled and concentrated in a 3 K Macrosep centrifugal concentrator (Pall Gelman Laboratory, Ann Arbor, MI). The concentrated fraction was chromatographed on a HiLoad 16/60 Superdex 75 prepgrade column (Amersham Biosciences) equilibrated with buffer A. The fractions containing EIIA^{Ntr}(H73A) were pooled and concentrated as described above. Purified protein was stored at –80°C until use.

Acetohydroxy acid synthase assay

The specific activity of AHAS was measured using the standard colorimetric assay for acetoin, which is formed from acetolactate after acid quench, as previously described (Singh *et al*., 1988; Epelbaum *et al*., 1990). Bacterial cells were disrupted by passage through a French pressure cell in a buffer containing 0.5 M KCl, 50 mM Tris-HCl, pH 7.5 and 20 μM flavin adenine dinucleotide (FAD). The enzymatic activity was assayed at 37°C in the presence of 40 mM pyruvate, 0.06 mM thiamine pyrophosphate, 0.06 mM FAD and 5 mM $MgCl_2$ in 10 mM Tris buffer, pH 7.5. The reaction mixture (0.5 ml) was incubated at 37°C for 40 min and the reaction was stopped by the addition of 0.05 ml 50% H_2SO_4. Incubation was continued for an additional 30 min at 37°C, in order to decarboxylate all the acetolactate formed during the incubation to yield acetoin. Aliquots (0.2 ml) of incubation mixtures were taken into tubes containing 0.36 ml distilled water, 0.4 ml of 0.5% creatine solution and 0.4 ml of 5% freshly made α-naphthol solution (prepared in 2.5 N NaOH), vortexed and incubated at 37°C for 15 min. Tubes were briefly vortexed and A_{540} was read against blanks to which H_2SO_4 was added before extract addition. Specific activity was expressed as μmoles acetoin formed per h per mg protein under the conditions stated, referenced to an acetoin standard curve. Total protein was determined colorimetrically, using bovine serum albumin as the standard.

Construction of ilvB–lacZ and ilvI–lacZ fusions and β-galactosidase assays

The DNA fragment covering from 1113 bp upstream to 167 bp downstream of the *ilvB* start codon was amplified by PCR using primers 5′-AGA ATA TGG AGG ATC CGC GTG CTT TGG CTT-3′ (an engineered BamHI site underlined) and 5′-AAT TAG AAT AAG TCA CTA CGA ATT CCC ATA-3′ (an engineered EcoRI site underlined). The PCR product, digested with EcoRI and BamHI, was inserted into pRS415 (Simons *et al*., 1987) to make pCR4. The sequence of the *ilvB* insert was verified by sequencing, and the fusion was transferred to λRS45 by *in vivo* recombination as described previously (Simons *et al*., 1987). Bacteriophage λ carrying the fusion was used to lysogenize JM101 at low multiplicity, and several lysogens were analysed to obtain a monolysogen (CR400). CR401 (CR400 *ptsN*::Tet^R) was constructed by P1

transduction of the Tet^R region of CR300. Similarly, the DNA fragment covering from 679 bp upstream to 121 bp downstream of the *ilvI* start codon was amplified by PCR using primers 5′-TGA TTA GTT ATG AAT TCT TCC ATA GTC CTG-3′ (an engineered EcoRI site underlined) and 5′-CGA CCA TCT CGG ATC CAG ACA ACA TCT CCA-3′ (an engineered BamHI site underlined) to make the *ilvI–lacZ* fusion construct and strains CR500 and CR501 (see Table 2). After CR401 and CR501 cells were grown in LB medium to the mid-logarithmic growth phase, cells were centrifuged, washed and resuspended in M9 minimal medium containing 0.5% glucose. After the nutritional downshift, β-galactosidase activity was measured at mid-logarithmic growth phase as described previously (Miller, 1972).

Reverse transcription polymerase chain reaction analysis

Total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA) from cells grown in M9 minimal medium containing 0.5% glucose to mid-logarithmic phase according to the manufacturer's instructions. Preparations were treated with RNase-free DNase I (Roche Applied Science, Indianapolis, IN) at 37°C for at least 4 h to eliminate contaminating DNA. The absence of contaminating genomic DNA in RNA preparations was verified by performing PCR. To synthesize cDNA, total RNA (2 μg) was subjected to RT reactions using Superscript RT (Invitrogen) using the following primers: *ilvB* forward, 5′-GTC CAC TTC CTG GAA GGC GTC GGT GCC GAT-3′; *ilvB* reverse, 5′-ATG GCA AGT TCG GGC ACA ACA TCG ACG CGT-3′; *ilvI* forward, 5′-GTG TTT ATG TCT CTG GCT GCC AAT TGC TTAA-3′; *ilvI* reverse, 5′-ATG GAA TGG AAT CCA TAT AAG CGG TGG CGA-3′; *lrp* forward, 5′-ATG GTA GAT AGC AAG AAG CGC CCT GGCA-3′; and *lrp* reverse, 5′-ACA TAT CCG GCA CGC GTG TTT TCA ACA GGT-3′. Amplification reactions were performed in a GeneAmp PCR System for 5 min at 94°C, followed by 30 or 40 cycles of 94°C for 20 s, 55°C for 30 s and 72°C for 1 min kb^{-1}, concluding with extension at 72°C for 4 min.

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