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Proteome analysis of factor for inversion stimulation (Fis) overproduction in *Escherichia coli*

The factor-for-inversion stimulation protein (Fis) is a global regulatory protein in *Escherichia coli* that activates ribosomal RNA (rRNA) transcription by binding to three upstream activation sites of the rRNA promoter and enhances transcription 5- to 10-fold *in vivo*. Fis overexpression results in different effects on cell growth depending on nutrient conditions. Differential proteome analysis of Fis-expressing cells shows ten protein spots corresponding to Fis overexpression in both rich (YT) and minimal (M9+glucose) media. Three of these spots have been identified as elongation factor TS, histidine-binding periplasmic protein precursor, and ketol-acid reductoisomerase.

1 Introduction

The desire to control recombinant protein production in bacterial cell culture necessitates an understanding of the control of ribosome synthesis. The coordination of ribosome synthesis is a complex physiological task for cells and it is known that the regulation of ribosomal RNA (rRNA) synthesis is a critical determinant within this process [1]. Indeed, ribosomes and associated factors can account for up to 50% of cell mass at high growth rates [2] while rRNA constitutes over half of the total cellular RNA in cells under these same conditions [3]. *Escherichia coli* has seven rRNA operons (*rrn* operons) in its genome and the synthesis of most (not all tRNAs are cotranscribed with rRNA) transfer RNAs (tRNA) is coregulated with that of rRNA by cotranscription. Variation in rRNA expression appears as a consequence of nutrient conditions which affects growth rate. In particular, the number of ribosomes and the synthesis rates of rRNA are roughly proportional to the growth rate squared (μ^2) and rRNA synthesis is the rate-limiting step in ribosome synthesis in *E. coli* [4]. Furthermore, it is known that rRNA expression is feedback-regulated by growth rate control [5]. Experiments show that this feedback regulation depends on the number of actively translating ribosomes [6, 7]; however, the mechanism by which translating ribosomes are coupled to growth control has not been completely elucidated. rRNA expression is further, but separately, regulated by a number of repressing and activating mechanisms such as

ppGpp levels as part of the stringent response [8]. The *rrn* operon contains upstream promoter sites which are responsible for strong stimulation of promoter activity but are not required for growth rate regulation. These sites include binding sites for the factor-for-inversion stimulation (Fis) protein.

Fis is an 11.2 kDa, p*I* 9.34, relatively abundant DNA-binding protein. Large fluctuations in Fis expression occur during the growth cycle, with Fis becoming less abundant in late-log-phase cells [9, 10]. Fis is also less abundant in cells grown on poor medium and this scarcity appears to cause a decrease in activity of most growth rate-regulated promoters of stable rRNA operons [11, 12]. Fis has the ability to stimulate site-specific DNA inversion reactions by binding to an enhancer sequence and bending the DNA [13, 14]. Although Fis is not required for cell growth [15], it has been shown to stimulate stable rRNA synthesis both in rich medium and under conditions of nutrient upshift [16]. Furthermore, Fis-dependent activation is crucial for providing the high rate of rRNA synthesis required forrapid cell growth. We have previously studied the effect of Fis overproduction on growth and ribosome synthesis for different media (submitted). Fis-expressing cells demonstrate significant different effects on growth characteristics on rich media and minimal media. Here, we employ the tools of proteome analysis to help elucidate the biochemical basis for this phenomena.

2 Materials and methods

2.1 Strains and vectors

Wild-type Fis was expressed in *E. coli* W1485 △H LAM-rpoS396(Am) rph-1 [17]. A positive mutant of Fis used in this study is designated GS. The GS gene contains a G-to-S substitution at amino acid 72 of the wild-type Fis protein which renders the GS protein much less able to activate expression of the *rrnB* promoter as compared to

wild-type Fis [18]. Both the Fis and GS genes were subcloned into the control of the *lac-tac* promoter for isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression. Cells were cultivated at 37°C on yeast-tryptone (YT) media or minimal M9 media supplemented with 0.2% glucose or 0.2% glycerol [19]. OD measurements were made on a Beckman DU 640 spectrophotometer (Palo Alto, CA).

2.2 Proteome analysis

2.2.1 Sample preparation and electrophoresis

Samples for proteome analysis were taken at approximately 3 h post-induction with 0.25 mM IPTG. The culture was pelleted and washed four times in a solution containing 3.0 mM KCl, 1.5 mM KH_2PO_4, 68 mM NaCl, and 9.0 mM NaH_2PO_4. The washed pellet was resuspended in a solution containing 10 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM Pefabloc SC (Boehringer, Indianapolis, IN) and 0.1% SDS. This solution was sonicated at full power on ice for 30 s in a Fisher Model F550 sonifier (Pittsburgh, PA) and stored at −75°C until use. One hundred μg of protein (corresponding to 40 μL of sample) was mixed with 20 μL of 8 M urea, 4% w/v CHAPS, 65 mM DTT, 67 mM Tris, pH 8.0, and a trace of bromophenol blue. This mixture was loaded onto pH 3–10 nonlinear Immobiline gels (Amersham-Pharmacia-Hoefer, Piscataway, NJ) by in-gel rehydration. The reswelling solution contained 8 M urea, 2% CHAPS, 0.3% DTT, 1.33% BioLyte 3–10 and 0.67% BioLyte 5–7 (Bio-Rad Laboratories, Richmond, CA), and a trace of bromophenol blue. Isoelectric focusing was performed for 71 750 Vh. Gels were subsequently equilibrated for 15 min in a solution containing 6 M urea, 2% DTT, 30% glycerol, 2% SDS and 0.05 M Tris, pH 6.8, and for 5 min in a solution containing 6 M urea, 2.5% iodoacetamide, 30% glycerol, 2% SDS and 0.05 M Tris, pH 6.8. Strips were transferred to a vertical SDS-PAGE tank (Bio-Rad) and covered with a solution of 0.5% agarose, 25 mM Tris (pH 8.3), 198 mM glycine, and 0.1% SDS. The 1.5 mm thick 12%T gels [20] were run at 40 mA per gel until the dye front migrated to the end of the gel. Gels were stained with ammoniacal silver as described previously [21].

2.2.2 Computer-assisted analysis

Computer-assisted gel analysis (Melanie II, Bio-Rad Laboratories) was performed on images captured with a Molecular Dynamics Personal Densitometer. Default parameters were used for feature detection and matching and corrected by visual inspection. An estimate of the relative quantitative changes was made based on the change in percent volume among silver-stained gels. These quantitative data were obtained from multiple gel runs and from a Melanie II analysis of the gel images. Data from spots that were very faint or very strongly stained were not included in quantitative comparisons. Spot changes of interest were tested on multiple gels for reproducibility. The genetic basis for spot changes was made based on a comparison to the *E. coli* 2-DE database available at SWISS 2-D PAGE (www.expasy.ch). It is noted that existing *E. coli* databases use strain W3110 whereas this study was performed in strain W1485. Some strain-specific differences in these proteomes exist and *N*-terminal sequence tagging of landmark proteins was performed to help establish reference points for comparison between W1485 and W3110 proteomes.

3 Results and discussion

3.1 Growth studies

Fis-expressing cells were grown in YT media containing 0.25 mM IPTG and compared to GS-expressing cells (GS-expressing cells are positive mutants of Fis) grown in the same media. GS-expressing cells have a post-induction growth rate of 0.119 h^{-1} while Fis-expressing cells demonstrate a post-induction growth rate of 0.100 h^{-1} (see Fig. 1 and Table 1). This 16% decrease in growth
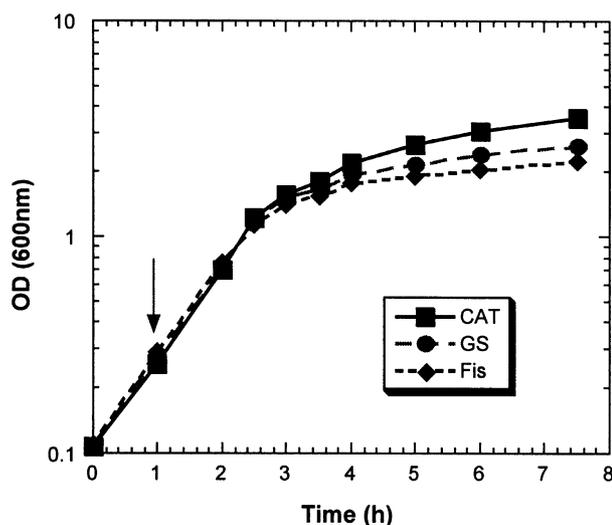

Figure 1. Growth curves for Fis-, GS-, and CAT-expressing *E. coli* grown on YT medium. The arrow indicates the time of IPTG-induced protein induction.

Table 1 Post-induction growth rates (h^{-1}) of Fis- and GS-expressing cells on different culture medium

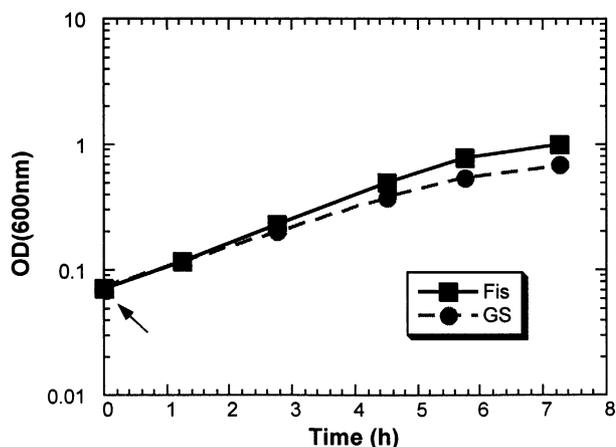|	YT 0.25 mM IPTG	M9+glycerol 0.25 mM IPTG	M9+glucose 0.25 mM IPTG
GS	0.119	0.245	0.319
Fis	0.100	0.165	0.389
Change	16% decrease	33% decrease	22% increase

Figure 2. Growth curves for Fis- and GS-expressing *E. coli* grown on minimal M9 + glucose medium. The arrow indicates the time of IPTG-induced protein induction.

Figure 3. Growth curves for Fis- and GS-expressing *E. coli* grown on minimal M9 + glycerol medium. The arrow indicates the time of IPTG-induced protein induction.

rate parallels an expected increase in rRNA synthesis as measured directly by primer extension (data not shown). Elevated rRNA synthesis is an expected observation based on the known function of Fis. In contrast, CAT-expressing control cells also grew 40% faster than Fis-expressing cells on YT media (0.167 h^{-1}) and cell growth was proportionally inhibited at higher levels of gene induction (data not shown).

Fis-expressing cells grown on minimal M9 media supplemented with glucose demonstrate enhanced growth characteristics compared to GS-expressing cells (see Fig. 2 and Table 1). The Fis-expressing cells, 3 h post-induction, grow 22% faster than GS-expressing cells (0.319 h^{-1} *versus* 0.389 h^{-1}). Here again Fis-expressing cells have elevated rRNA synthesis as expected. In contrast, Fis-expressing cells grown on M9 media supplemented with glycerol (see Fig. 3) demonstrated a 33% decrease in growth rate as compared to GS-expressing controls (0.245 h^{-1} *versus* 0.165 h^{-1}). A decrease in growth rate is also observed for these cells growing on YT medium. This observation suggests a link between the phosphotransferase system (PTS) and Fis activity in cells. When glucose is present in the medium, intracellular cyclic adenosine monophosphate (cAMP) levels are relatively low and the PTS system is active; however, when glucose is not present, cAMP levels are relatively high and the PTS system is inactive. The observed growth rate differences are consistent with low intracellular cAMP levels in cells grown on glucose-supplemented medium. It is known that several Fis-regulated promoters contain putative cAMP-cAMP receptor protein (CRP) binding sites (CAP binding sites) overlapping the Fis-binding sites [22]. It is possible that a depletion in intracellular cAMP enables Fis to bind more
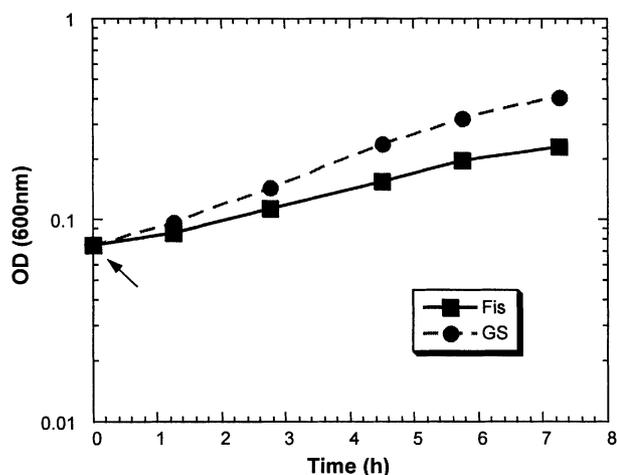
readily to these promoters, thus activating these pathways and enabling faster cell growth (compared to GS-expressing cells).

3.2 Proteome analysis

The presence of various nutrients in YT-rich medium (as compared to minimal M9 medium) suggests that *E. coli* grown on YT will demonstrate a different physiology from their M9 counterparts. In particular, YT-grown cells will need to retrieve various amino acids and other nutrients from the media for incorporation into biomass; yet these cells will not need to synthesize these molecules from precursors. In contrast, cells grown on M9 media will actively synthesize many molecules from precursors while devoting significant effort to retrieve a particular carbon source from the medium. To elucidate how these physiological differences may contribute to growth-rate differences seen among the Fis-expressing strains, we performed two-dimensional protein electrophoresis on samples obtained from Fis-expressing and GS-expressing cells. Cells grown in YT media (Figs. 3 and 4) were compared, as were cells grown in minimal M9+glucose medium (Figs. 6 and 7).

A direct comparison between Fis-expressing and GS-expressing strains grown on YT media and at 3 h post 0.25 mM IPTG induction reveals the presence of approximately 1600 protein spots. Of these, we observe 23 spots which are qualitatively different or quantitatively upregulated at least 2-fold in Fis-expressing cells (see Fig. 4) as compared to GS-expressing cells (see Fig. 5) and six spots which are likewise different in GS-expressing cells as compared to Fis-expressing cells. These differences
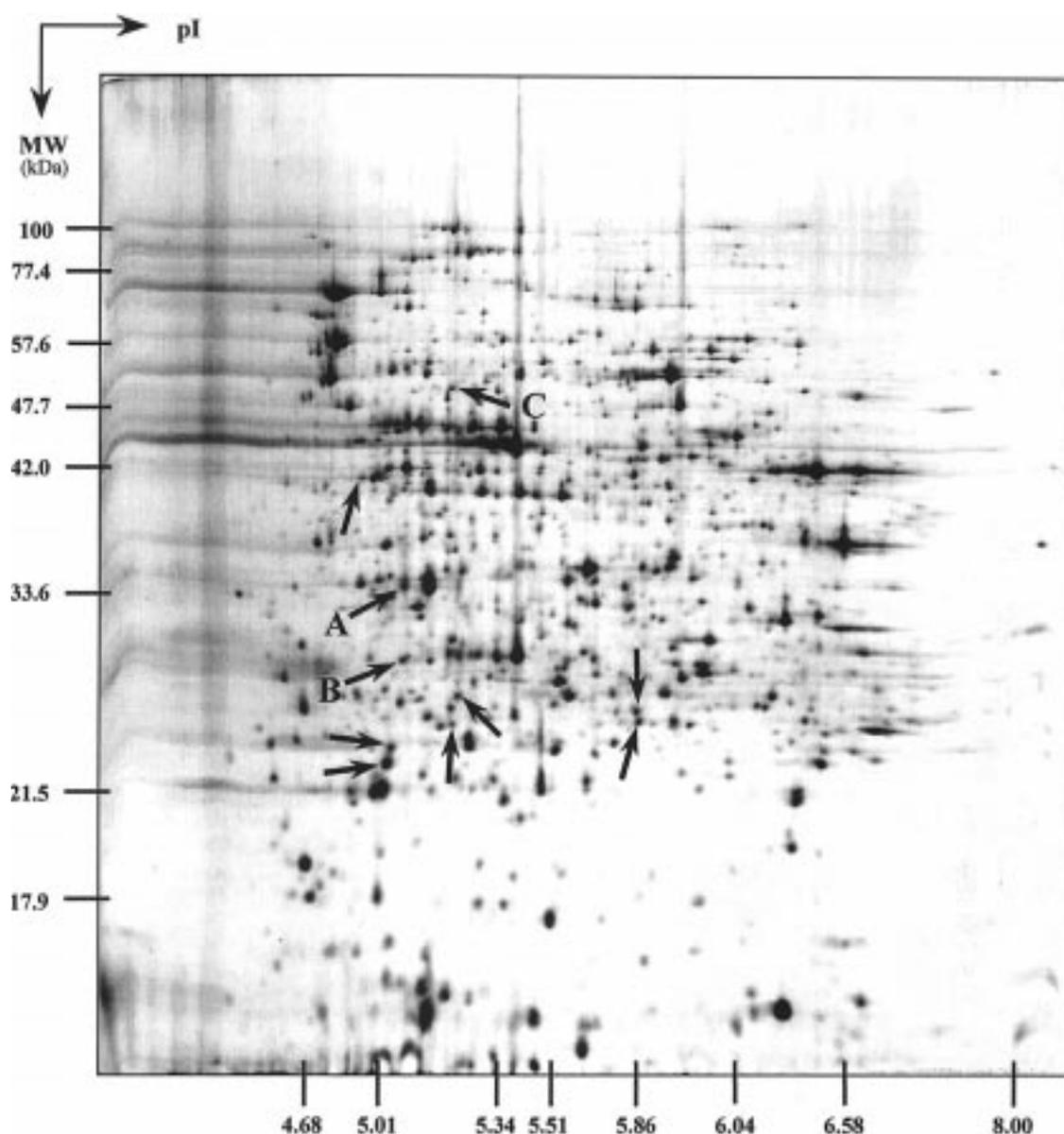
Figure 4. Proteome of Fis-expressing *E. coli* grown on YT medium. The location of 10 spots that appear to be involved in the observed decrease in growth rate on YT medium and the observed increase in growth rate on M9 medium are noted. Three of these 10 spots have been characterized as A, elongation factor TS; B, histidine-binding periplasmic protein precursor; and C, ketol-acid reductoisomerase.

can be attributed to the presence or absence of Fis-binding and subsequent gene activation, which results in the observed decrease in growth rates for Fis-expressing cells on YT medium.

Fis-expressing cells (see Fig. 6) were compared to GS-expressing cells (see Fig. 7) grown on M9 media supplemented with glucose. The Fis-expressing cells demonstrate enhanced growth rate as compared to the GS-expressing cells. In this comparison 19 spots appear significantly upregulated in Fis-expressing cells as compared to GS-expressing cells while only one spot has that property in GS-expressing cells. Those spots which appear in Fis-expressing cells and not in GS-expressing cells grown on both YT media and M9 media likely correspond to genes which demonstrate enhanced expression due to increased Fis-expression. There are ten spots with this characteristic (as labeled on Figs. 4 and 5). These genes, and their corresponding pathways, are thus related to the observed differences in growth characteristics on M9 and YT media. To date, we have characterized three of these ten spots. They are: elongation factor TS
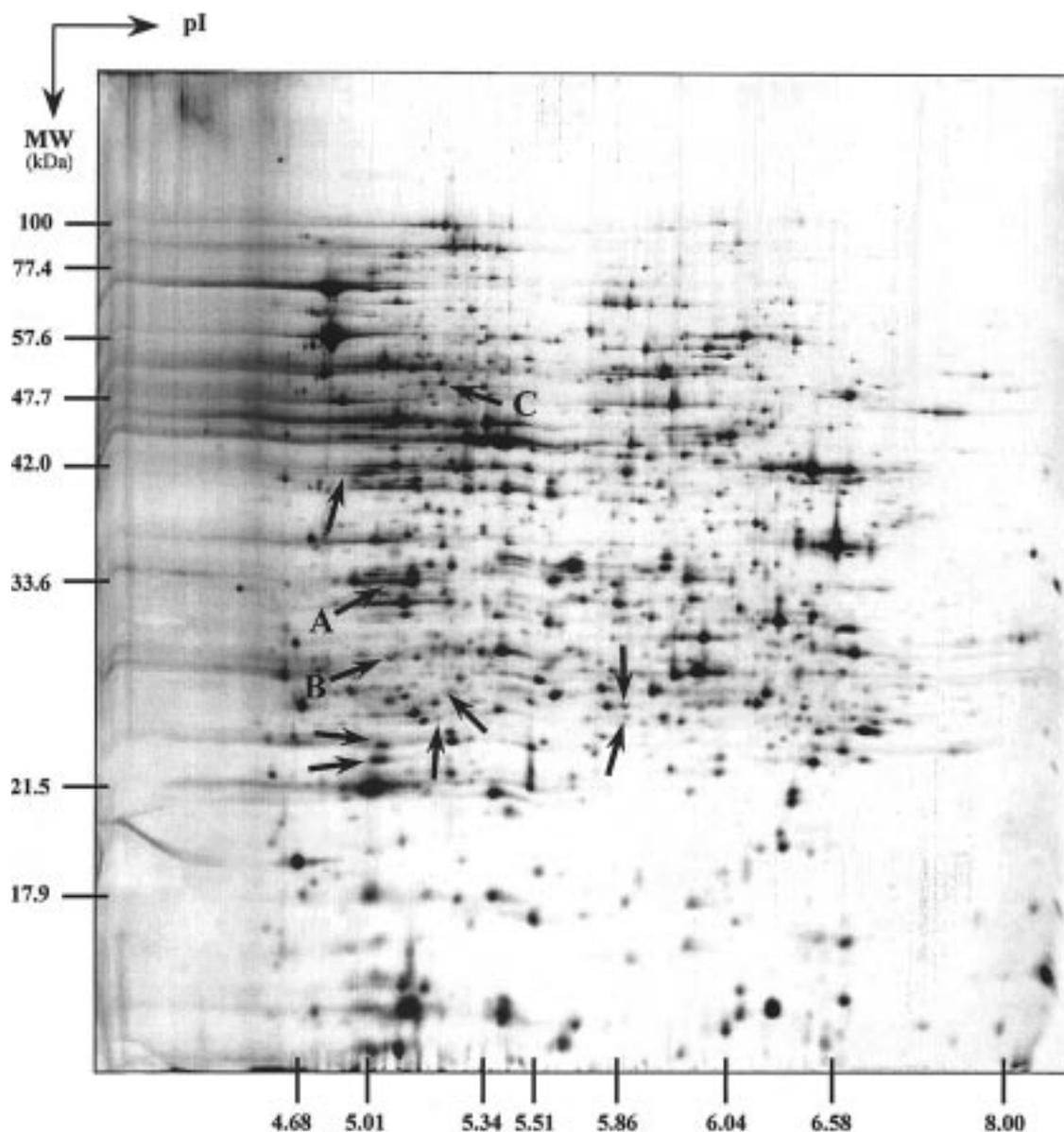
Figure 5. Proteome of GS-expressing *E. coli* grown on YT medium.

(EFTS; SWISS-PROT Accession No. P02997), histidine-binding periplasmic protein precursor (HisJ; SWISS-PROT Accession No. P39182), and ketol-acid reductoisomerase (IlvC; SWISS-PROT Accession No. P05793). We are in the process of characterizing the other changes of interest which are not present in current databases.

3.3 Characterized spot changes

Elongation factor TS associates with elongation factor-Tu and forms part of the ribosomal complex. As such, its elevated expression in Fis-expressing cells is expected because Fis activates the rRNA promoter. The histidine-binding periplasmic protein precursor is a component of the high-affinity histidine permease, which is a binding-protein-dependent transport system. The family of proteins which make up this transport system (including HisJ) has been implicated in the active transport of amino acids and sugars. Elevated expression of this protein precursor in Fis-expressing strains leading to different growth characteristics on media with and without glucose suggests a nonlinear relationship between amino acid import and the PTS uptake system. Ketol-acid reductoisomerase performs the second step in valine and isoleucine biosynthesis. Elevated expression of this protein is also consistent with the observed phenomena because cells grown on
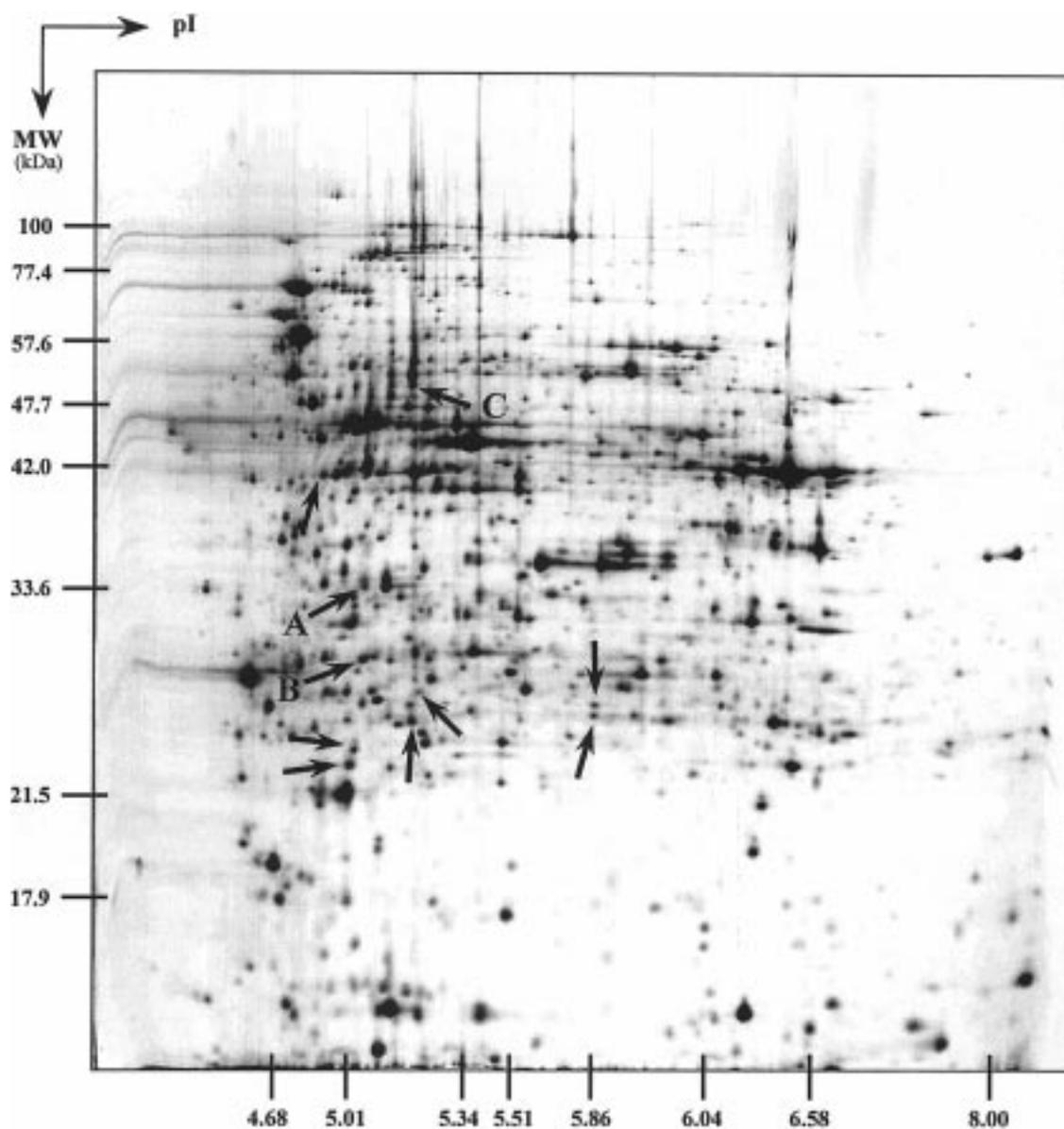
Figure 6. Proteome of Fis-expressing *E. coli* grown on M9+glucose medium. Nineteen spots are significantly increased as compared to GS-expressing cells. The location of 10 of these is depicted on Fig. 4.

M9 media must synthesize valine and isoleucine for incorporation into biomass. Fis-expressing cells grown on YT medium can recruit these amino acids from the medium.

4 Concluding remarks

In an attempt to elucidate the biochemical coordination of rRNA synthesis and growth rate control we have studied the effects of Fis-overexpression on *E. coli* physiology under different growth conditions. Differential proteome analysis suggests several key changes which involve various aspects of cellular physiology including carbon metabolism, nutrient uptake, and translation among others. We are currently investigating the genetic basis for uncharacterized spot changes as well as developing a predictive model for use in the engineering of cells with enhanced recombinant protein production.
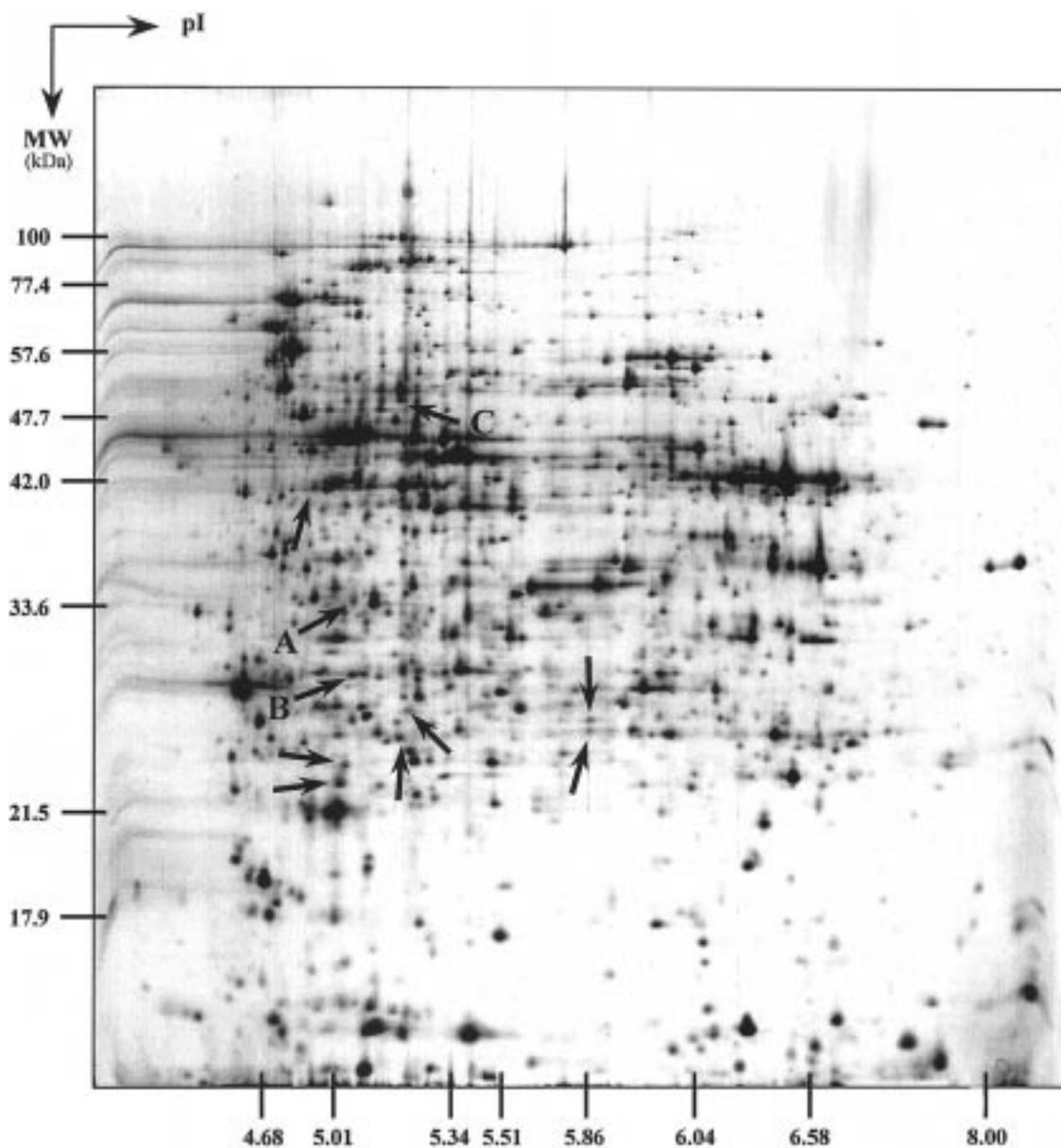
Figure 7. Proteome of GS-expressing *E. coli* grown on M9+glucose medium.

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