Expression levels of transcription factors in *Escherichia coli*: growth phase- and growth condition-dependent variation of 90 regulators from six families

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The expression pattern of the genome in *Escherichia coli* is controlled by regulating the utilization of a limited number of RNA polymerases between a total of 4600 genes on its genome. The distribution pattern of RNA polymerase on the genome changes after two steps of protein–protein interaction with seven sigma subunits and about 300 transcription factors (TFs). Based on a systematic search for the regulation target promoters recognized by each TF, we propose two novel concepts: each TF regulates a number of target promoters; and each promoter is regulated by many TFs. In parallel, attempts have been made to determine the intracellular concentrations of all TFs using two systems: quantitative immunoblot analysis using TF-specific antibodies; and reporter assay of TF promoter activities. The direct measurement of TF protein level has so far been published for a set of 60 regulators with known functions. This study describes the determination of growth phase-dependent expression levels of 90 TFs using the reporter assay system. The translational fusion vector was constructed from the TF promoter sequence including an N-terminal proximal TF segment and the reporter GFP. At the beginning of cell growth, high-level expression was observed only for a small number of TFs. In the exponential phase, approximately 80% TFs are expressed, but the expressed TF species change upon transfer to the stationary phase. Significant changes in the pattern of TF expression were observed between aerobic and anaerobic conditions. The list of intracellular levels of TFs provides further understanding to the transcription regulation of the *E. coli* genome under various stressful conditions.

INTRODUCTION

Single-cell bacteria are directly exposed to frequently changing environments in nature and thus carry sophisticated genetic systems for adaptation to environmental changes (Ishihama, 2010, 2012; Yamamoto, 2014). On the basis of the complete genome sequence of several model *Escherichia coli* strains, the whole set of about 4600 genes has been predicted to exist in *E. coli* K-12 (Hayashi et al., 2006; Riley et al., 2006). In growing *E. coli* cells under laboratory culture conditions, only one-quarter to one-third of the genes on its genome are expressed, the others remaining silent. The majority of these uncharacterized silent genes must be expressed and utilized for adaptation and survival of *E. coli* under stressful conditions. Thus, even for this well-characterized model organism, the gene functions remain unidentified or unpredicted for approximately one-quarter because expression conditions of these silent genes have not yet been established.

As the total number of RNA polymerases (RNAPs) in *E. coli* K-12 is less than the total number of genes on its genome (Ishihama, 2000), we proposed a model in which the change in genome transcription pattern takes place mainly through controlling the utilization of this limited number of transcription apparatuses between 4600 genes on the genome (Ishihama, 2010, 2012). Two groups of regulatory proteins, sigma factors and transcription factors (TFs), are involved in modulation of the gene selectivity of RNAP. In the first step, the sigma subunit binds to the RNAP core enzyme and provides it with the promoter recognition activity, leading to formation of the holoenzyme. In *E. coli* K-12, seven species of the sigma subunit exist, each recognizing a specific set of promoters. Some of the recognition target genes have been characterized for each sigma subunit using standard molecular genetics approaches
The gene selectivity of holoenzymes is further modulated through interaction with the second group of regulatory proteins, the TFs. The decision regarding gene utilization is therefore executed not only by the sigma factors but also by the TFs. In _E. coli_ K-12, about 300 species of TF have been identified (Pérez-Rueda & Collado-Vides, 2000; Ishihama, 2010). In the classic model of molecular genetics, transcription of one gene or operon was considered to be under the control of one gene-specific TF while each TF was believed to regulate one specific target gene or operon (Martínez-Antonio & Collado-Vides, 2003; Babu _et al._, 2004; Browning & Busby, 2004). Extensive efforts have recently been devoted to identifying the regulation target genes under the control of each TF by using high-throughput experimental systems such as the transcriptome and RNA-seq analyses of genome transcription patterns of TF-defective _E. coli_ mutants (Cho _et al._, 2009; Raghavan _et al._, 2011; Ginnoukos _et al._, 2012) and upon exposure to stressful conditions such as changes in nutrients (Oh _et al._, 2002), exposure to heat shock (Richmond _et al._, 1999) or cold shock (Phadtare & Inouye, 2004), the presence of hydrogen peroxide (Zheng _et al._, 2001) or external metals (Lee _et al._, 2005), anaerobic conditions (Overton _et al._, 2006) and within biofilm (Ren _et al._, 2004). Direct measurement of TF-associated sites in _vivo_ along the _E. coli_ genome has been performed by ChIP-chip analysis (Herring _et al._, 2005; Grainger & Busby, 2008; Mooney _et al._, 2009). These _in vivo_ analyses together provide information regarding TF-associated sites on the _E. coli_ genome under the culture conditions employed, but are unable to identify the whole set of TF recognition sites because the functional forms of TFs are not always present in _E. coli_ under laboratory culture conditions (see below) and because the TF-binding sites are often masked by other DNA-binding proteins.

For the identification of direct targets by each TF, we have developed two _in vitro_ screening systems: an improved method of the Genomic SELEX screening system (Shimada _et al._, 2004); and the promoter-specific TF screening system (Shimada _et al._, 2013). Results so far obtained using these two lines of research indicate that: (1) a single TF is generally involved in the regulation of several genes and (2) one promoter is often regulated by several TFs. The number of regulation targets by each TF is much higher than those listed in databases such as RegulonDB and EcoCyc (Keseler _et al._, 2013; Salgado _et al._, 2013). For instance, the _csgD_ gene encoding the master regulator of biofilm formation is regulated by as many as 30 TFs (Ogasawara _et al._, 2010; Ishihama, 2012). More than 15 TFs are involved in regulation of the _sdiA_ gene encoding the regulator for cell division and differentiation (Shimada _et al._, 2013). On these multifactor-regulated promoters, the positive and negative TFs compete with each other for effective binding to a narrow region of a single and the same promoter, but in addition, collaborative interplay takes place between positive TFs and between negative TFs (Ogasawara _et al._, 2010; Ishihama, 2012).

Once we identify the regulation targets of each TF, it is possible to predict the transcription pattern of the genome if we understand the intracellular concentrations of these factors. At present, however, only fragmentary information is available on the intracellular concentrations of TFs [for instance, TrpR level by Gunsalus _et al._ (1986); Lrp level by Borst _et al._ (1996)], but the systematic determination of TF levels has not previously been performed. Moreover, for the accurate estimation of intracellular levels of the regulatory proteins for transcription, all the test proteins must be measured using the same cultures of the same bacterial strains and the same experimental systems. We then systematically measured intracellular concentrations of sigma subunits and TFs using two approaches: quantitative immunoblot analyses using anti-sigma (Jishage & Ishihama, 1995; Jishage _et al._, 1996; Maeda _et al._, 2000) or anti-TF antibodies (Ishihama, _et al._, 2014); and quantification of promoter activity using the promoter assay vector of the fluorescent protein reporter system (Shimada _et al._, 2005). In this paper, we describe the determination of intracellular levels of 90 TFs of six _TF_ families by using the TF promoter–GFP translation fusion vectors. The result increases our understanding of the regulation of genome transcription in _E. coli_ under various stressful conditions in nature.

**METHODS**

**Bacterial strains and plasmids.** _E. coli_ K-12 W3110 type-A (Jishage & Ishihama, 1997) was used for measurement of RNAP subunits and TFs throughout this study. Cells were grown in Luria–Bertani (LB) medium at 37 °C under aeration with constant shaking at 140 r.p.m. or under anaerobic conditions within an anaerobic chamber (COY Laboratory Product). Cell growth was monitored by measuring the turbidity at 600 nm with a TVS062CA photometer (Advantec).

**Construction of the assay vector of the TF promoter–GFP translational fusion.** DNA sequences of each TF gene between approximately –500 and +150 with respect to the initiation codon, including the promoters and the N-terminal proximal TF-coding sequences, were PCR-amplified using pairs of the respective primers containing _BglII_ and _EcoT22I_ sites. PCR-amplified sequences were treated with _BglII_ and _EcoT22I_ and inserted into pGRF vector, the promoter assay vector of the GFP reporter (Shimada _et al._, 2004), between _BglII_ and _EcoT22I_ (Fig. 1). The resulting plasmids encode TF peptide–GFP fusions.

**Measurement of TF expression.** The expression level of TF genes was determined by measuring the level of TF promoter-driven TF–GFP translational fusions. Plasmids containing the TF peptide–GFP fusions were transformed into _E. coli_ W3110 type-A. Transformants were grown in LB medium at 37 °C under either aerobic conditions with shaking or anaerobic conditions within the anaerobic chamber. At various growth phases, aliquots were subjected to measurement of GFP fluorescence using the FACS Calibur system (BD Biosciences). For detection of GFP fluorescence of cells grown under anaerobic conditions, cells were stored for 30 min on ice prior to FACS analysis. FACS patterns were analysed using a total of 100,000 cells, and four parameters (Mean value, GeoMean value, Median value and PeakCh value) were determined. A low level of fluorescence was detected for
untransformed cells. As this background was negligible for the PeakCh value, and because a good correlation was detected between the Mean value and the PeakCh value, the promoter activity described was determined using the PeakCh value. This measurement of TF promoter-driven GFP expression was performed at least twice for each experimental system using independent cultures. The difference between two determinations was less than 12%.

RESULTS AND DISCUSSION

Determination of the expression levels of TFs: overall research strategy and tactics

For understanding the transcription regulation of the *E. coli* genome as a whole, it is necessary to determine the intracellular concentrations of all seven sigma factors and all 300 TFs. The intracellular concentrations of all seven sigma factors in growing cells of *E. coli* W3110 have been determined (Jishage & Ishihama, 1995; Jishage et al., 1996; Maeda et al., 2000). Since then we have been involved in determination of the intracellular concentrations of the 300 TFs using two approaches: (1) quantitative immunoblot analysis and (2) reporter assay of promoters of the genes encoding TFs. The first report of results using the quantitative immunoblot system has been published, which describes the intracellular concentration of 60 species of TFs with known regulatory functions (Ishihama et al., 2014). As a quick and accurate means of monitoring the expression level of a number of TFs in *E. coli* cells under various growth conditions, we employed in this study the second method, in which the expression level of TFs was determined using a modified version of the promoter assay vector (Shimada et al., 2004). As the synthesis of regulatory proteins is often controlled at the level of translation (for instance see Gottesman, 2004), we constructed the TF promoter assay vector as translational fusions between the reporter GFP and the test TF sequences from the entire promoter segment (about 500 bp sequence upstream of the initiation codon) down to the N-terminal proximal short segment (approx. 150 bp sequence) of the TF ORF (Fig. 1).

About 300 TFs in *E. coli* have been classified into 45 families based on protein structure (Ishihama, 2010), of which the most abundant family is LysR, which includes 45 members. In this study we examined 90 TFs (53 characterized TFs and 37 uncharacterized TFs) (Table S1, available in the online Supplementary Material) using this TF promoter–GFP translational fusion system. The collection analysed in this study included TFs from six families: AraC (18 member TFs), DeoR (five TFs), GntR (19 TFs), IclR (four TFs), LysR (37 TFs) and Xre (eight TFs). Among the 53 TFs with hitherto identified regulatory functions, the major groups of 34 TFs are involved in regulation of the genes for metabolism. A group of 11 TFs are involved in the control of cell division, cell shape and cell behaviour while another eight TFs are involved in response to external stress signals. This set includes two bifunctional nucleoid proteins, Dan (DNA-binding protein under anaerobic conditions) and Rob (right oriC binding protein).

Expression levels of TFs under aerobic conditions

Each of the 90 promoter assay vectors for the TF genes was transformed into *E. coli* W3110 type-A. Overnight culture of the transformant was transferred into fresh LB medium and grown at 37°C under aerobic conditions. The level of GFP was measured at lag phase (early growth phase), exponential growth phase, and stationary phase. The measurement was repeated twice using two independent cultures.

Lag phase. In the lag phase, a detectable level expression of TF promoter-directed GFP was observed only for 19 TFs (about 20%) (Fig. 2a; for details see Table 1, aerobic lag phase), implying that these TFs have unique roles in transcription of a set of genes needed for restart of cell growth from the dormant state. A high level of expression was detected only for SoxS, a dual transcriptional activator, which participates in the removal of superoxide and nitric oxide for protection of *E. coli* cells against superoxide-generating agents (Demple, 1996). The response of SoxS is triggered by activation of a sensor SoxR that contains two essential [2Fe–2S] clusters (Gaudu & Weiss, 1996). The activity of SoxR is switched off by removal of [2Fe–2S] centres and activated by their reinsertion. The two-step pathway of SoxRS activation constitutes a unique and sensitive system enabling cells to induce rapidly a protective response to a broad range of oxidative stresses in nature. The SoxS regulon overlaps with those of the homologous TFs, MarA and Rob, of the AraC family.
The MarA–SoxS–Rob regulon is involved in antibiotic resistance, superoxide resistance and tolerance to organic solvents. Many genes involved in response to external stresses are activated by all three TFs, each responding to different signals: superoxides for SoxS, aromatic weak acids such as salicylate for MarA, and bile salts and dipyridyl for Rob (Demple, 1996; Martin & Rosner, 2001). Although SoxS is induced in the lag phase of cell growth, both MarA and Rob are not induced (Table 1), indicating that SoxS plays a unique role in the initial stage of cell growth. More than 25 operons have been identified in the SoxS regulon (Salgado et al., 2013), of which five are regulated by SoxS but not by MarA or Rob. Expression of these SoxS-specific targets, PstG (glucose PTS permease),FldA (flavodoxin 1), FldB (flavodoxin 2), Pgi (phosphoglucone isomerase) and RibA (riboflavin synthesis), are all involved in the generation of metabolic energy for restart of cell growth from the resting state.

**Fig. 2.** Growth phase-dependent expression of TFs in *E. coli* cells grown under aerobic conditions. TF promoter assay vectors were transformed into *E. coli* WS110 type-A, and grown in LB at 37 °C under aerobic conditions. TF promoter-directed expression level of TF–GFP fusion proteins was measured at various growth phases: (a) lag phase, (b) exponential phase and (c) stationary phase. Test TFs are aligned in decreasing order of their expression levels. TFs are classified based on their expression level and are shown by orange colour gradient.

(Martin & Rosner, 2001). The MarA–SoxS–Rob regulon is involved in antibiotic resistance, superoxide resistance and tolerance to organic solvents. Many genes involved in response to external stresses are activated by all three TFs, each responding to different signals: superoxides for SoxS, aromatic weak acids such as salicylate for MarA, and bile salts and dipyridyl for Rob (Demple, 1996; Martin & Rosner, 2001). Although SoxS is induced in the lag phase of cell growth, both MarA and Rob are not induced (Table 1), indicating that SoxS plays a unique role in the initial stage of cell growth. More than 25 operons have been identified in the SoxS regulon (Salgado et al., 2013), of which five are regulated by SoxS but not by MarA or Rob. Expression of these SoxS-specific targets, PstG (glucose PTS permease), FldA (flavodoxin 1), FldB (flavodoxin 2), Pgi (phosphoglucone isomerase) and RibA (riboflavin synthesis), are all involved in the generation of metabolic energy for restart of cell growth from the resting state.

**Exponential phase.** In the exponential phase of cell growth, detectable levels of promoter activity were identified for 75 TF genes (83%) among the 90 TFs examined (Fig. 2b; also see Table 1, aerobic exponential phase), but 15 species of TFs were not expressed under this aerobic culture condition in a rich LB medium. Approximately 17 TF species were highly expressed (Fig. 2b; note that the level of GFP expression is shown by red gradient in a decreasing order). The only highly expressed SoxS in the lag phase continued to be expressed in the exponential phase. In addition to SoxS, other two homologous members of AraC family stress response TFs, MarA and Rob, were also highly expressed in this stage.

Likewise, two acid-response regulators, GadX and GadW, forming the principal acid resistance system in *E. coli* (Tramonti et al., 2002) were highly expressed even under apparently steady-state growth conditions (Table 1). This glutamate-dependent acid-response GAD system regulates
a set of acid-induced genes for acid resistance and a group of genes for multidrug efflux (Tuck et al., 2003). In addition, EnvY, a transcriptional regulator of several genes encoding cellular envelope proteins at low temperatures and during stationary phase (Lundrigan & Earhart, 1984), was also highly expressed in the exponential phase. One unexpected finding is that the stress response TFs including SoxS, MarA, GadX, Rob, EnvY and GadW (in decreasing order) are present at considerable levels even apparently under steady-state growth conditions in the absence of stress, probably because these TFs are stored in inactive forms for instantaneous response to external stresses or because these TFs regulate some as-yet-identified genes necessary for growth under the conditions herein employed.

A set of TFs for regulation of metabolic pathways such as ChbR, FabR, EutR, XylR and PuuR (in decreasing order) are also highly expressed (Fig. 2b; also see Table 1, aerobic exponential phase). ChbR is a dual transcriptional factor that regulates the chb operon involved in transport and degradation of chitobiase, a degradation product of chitin (Keyhani & Roseman, 1997). EutR regulates the transport of ethanolamine and virulence (Luzader et al., 2013). FabR is known to activate the genes for phenylacetate synthesis from 2-phenylethylamine (Hanlon et al., 1997). The fabR gene is under catabolic repression, and is highly expressed in the presence of succinate. XylR is a transcription factor involved in D-xylose degradation and utilization (Song & Park, 1997). PuuR is a transcription repressor that regulates transcription of several genes involved in putrescine utilization and transport (Kurihara et al., 2005). These findings raise the question of why the regulators for utilization of rather unusual carbon and nitrogen sources are induced in LB culture. The possibility that these TFs regulate as-yet-identified genes cannot be excluded. In fact, after genomic SELEX screening, we found that the number of regulation targets of E. coli TFs with known functions is generally higher than those hitherto identified (Ishihama, 2012).

High-level expression in the exponential phase was also found for a bitopic inner-membrane protein RodZ (Table 1), which is involved in the maintenance of cell shape through interaction with the MreB cytoskeleton (Shiomi et al., 2008). RodZ consists of an N-terminal helix–turn–helix domain and thus plays a role in binding to a specific set of mRNAs, including the type III secretion system, and modulates their translation (Mitobe et al., 2011). In addition, we have identified the binding of RodZ to a specific set of DNA sequences as determined by genomic SELEX screening (A. Ishihama, unpublished observations).

Stationary phase. Upon entry into the stationary phase, most of the TF genes are expressed but at decreased levels (Fig. 2c; also see Table 1, aerobic stationary phase). In particular, a marked reduction of expression levels was observed for a set of genes. For instance, the expression of SoxS and MarA, both highly expressed in the exponential phase, decreased in the stationary phase. In contrast, increased expression was identified for several TFs such as HdfR, McbR and NadR (Table 1). HdfR is a repressor of the genes encoding the flagellar master regulator FlhDC (Ko & Park, 2000) while McbR is a sensor of quorum sensing (QS) signal AI-2, and regulates biofilm formation and mucoidy by repressing expression of mcbA (Zhang et al., 2008). Together these two TFs participate in switching of the genomic programme from planktonic growth to biofilm formation. The TF–enzyme fusion protein NadR has both nicotinamide mononucleotide adenylyltransferase activity (Gerasimova & Gelfand, 2005), and regulates transcription of the genes involved in transport and de novo synthesis of NAD (Kurnasov et al., 2002). This bifunctional TF was induced in the stationary phase, implying that the metabolic system for energy production is altered upon entry into the stationary phase.

Expression levels of TFs under anaerobic growth conditions

The 90 transformants, each carrying one specific promoter–lacZ translational fusion, were also grown under anaerobic conditions using an anaerobic chamber. The level of LacZ expression was determined twice using two independent cultures. Overall, the expression pattern of TFs was significantly different between aerobic and anaerobic culture conditions (compare Figs 2 and 3; also Table 1).

Lag phase. When an overnight preculture was transferred into a fresh medium within an anaerobic culture chamber, the highest expression during the lag phase was observed for Dan (Fig. 3, aerobic lag phase; also see Fig. 4). Under anaerobic culture conditions, Dan is a major nucleoid protein in E. coli cells (Teramoto et al., 2010), forming the nucleoprotein filament for genome condensation (Lim et al., 2013). This finding indicates that the first response of E. coli upon exposure to an anaerobic environment is reforming of nucleoid confirmation supposedly for expression of the genes needed for adaptation to anaerobic conditions. The expression level of SoxS, the initial responder under aerobic culture conditions, decreased markedly under the anaerobic conditions.

Exponential phase. The expression of Dan, however, decreased in the exponential phase (Fig. 4), but another anaerobiosis-specific TF, TdcA, was highly induced (Fig. 4; also see Table 1), which participates in regulation of the tdc operon involved in transport and metabolism of Ser and Thr during anaerobic growth (Sawers, 2001). The metabolism of Ser and Thr biosynthesis forms a network of pathways linking not only other amino acids but also central primary metabolites for initiation of cell growth under anaerobic conditions (Sawers, 1998). In addition, the intracellular level increases for a set of TFs controlling metabolism under anaerobic conditions, including IclR, GcvA, XylR, EutR, ChbR, RhaR, FrlR and ExuR (Fig. 3b; for details see Table 1, anaerobic exponential phase), of which XylR, EutR and ChbR are also highly expressed in
**Table 1. Expression levels of TFs**

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the exponential phase under aerobic culture (Fig. 2b; also see Table 1, aerobic exponential phase). Other highly expressed TFs are also involved in switch control of metabolism for adaptation under anaerobic growth, including GcvA (glycine cleavage regulator) (Sawers, 2001) and TdcA (threonine dehydratase regulator) (Sawers, 2001) for amino acids, and FrlR (fructoselysine regulator) (Wiame et al., 2002), IclR (isocitrate lyase regulator) (Cortay et al., 1991) and RhaS (rhamnose regulator) (Egan & Schleif, 1993) for carbon sources (Fig. 4).

TFs involved in the switch of growth habit are also induced in exponential phase under the anaerobic conditions, including GadX, McbR, RodZ and SoxS. McbR is a sensor of QS signal AI-2, and regulates biofilm formation and mucoidity by repressing expression of mcbA (Zhang et al., 2008). McbA is involved in cell–cell contact and biofilm modulation.

**Stationary phase.** Under the anaerobic growth conditions, the expression pattern of TFs in the stationary phase is essentially the same as that in the exponential phase (Fig. 3b; also see Table 1, anaerobic culture), but NanR and QseB are exceptional, both being activated in the stationary phase. NanR regulates the genes involved in N-acetyl-neuramic
acid (or sialic acid) transport and metabolism and switching of type-1 fimbriation (Kalivoda et al., 2003). Likewise, the expression of QseD, one of the QS regulators, increases in the stationary phase under anaerobic culture conditions. QseB downregulates the flagella regulon, and thereby decreases motility (Habdas et al., 2010).

Differential expression of TFs between aerobic and anaerobic growth conditions

Among the 90 TFs examined in this study, a marked change in the expression pattern was observed between aerobic and anaerobic conditions for about 15 species. The first group of TFs are preferentially expressed only under aerobic conditions, including the oxidative stress response MarA–Rob–SoxS family TFs and the acid-response regulators GadX and GadW (Fig. 4a). Under aerobic conditions, the expression level was markedly reduced for these stress response TFs. Some of the aerobic condition-specific TFs also decreased or even disappeared under anaerobic conditions, including the stress response regulators HdfR, MarA and Rob (Fig. 4a). HdfR negatively regulates the expression of the flagellar master operon flhDC (Ko & Park, 2000), thereby leading to repression of flagella formation and inhibition of motility (Krin et al., 2010). The metabolism regulators GlcC and NadR also disappeared under anaerobic conditions. The known function of GlcC is regulation of the pathway of glycolate utilization (Pellicer et al., 1999).

By contrast, in the early stage of anaerobic growth, marked induction was observed for Dan, which participates in the refolding of nucleoid conformation (Fig. 4b) (Teramoto et al., 2010; Lim et al., 2013). The induction of Dan slowed quickly in the exponential phase, and instead a set of anaerobic condition-specific TFs were highly induced,
including FrlR, GcvA, IclR, RhaS and TdcA (Fig. 4b). These TFs regulate the genes involved in metabolism under anaerobic conditions.

The second group of TFs are expressed under both aerobic and anaerobic conditions. Note that the expression levels of TFs involved in regulation of the genes for metabolic enzymes, such as ChbR, DmlR, EutR, ExuR, RhaS and XylR (Fig. 4b; see also Table 1), remain fairly constant under both aerobic and anaerobic culture conditions. It is also noteworthy that, among the TFs with unidentified regulatory functions, high-level expression was observed in both aerobic and anaerobic conditions for YbaQ, YddM, YidL, YijO and YqhC (Table 1). These TFs may be involved in regulation of as-yet-unidentified genes for metabolic enzymes.

In conclusion, the expression of a set of TFs was found to change depending on the growth phase and between aerobic and anaerobic culture conditions. We thus propose that the level control of TFs is one of the major factors determining the utilization of a limited number of RNAPs and the expression pattern of the genome as a whole.

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