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NOTES

Induction of RpoS-Dependent Functions in Glucose-Limited Continuous Culture: What Level of Nutrient Limitation Induces the Stationary Phase of *Escherichia coli*?

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***treA* and *osmY* expression and RpoS protein levels were investigated in glucose-limited continuous culture. The level of induction of these stationary-phase markers became as high during growth at a *D* of 0.1 to 0.2 h^{-1} as in carbon-starved batch cultures but only in *rpoS*$^+$ bacteria. The stress protectant trehalose was actually produced at higher levels at low growth rates than in stationary-phase cultures. The pattern of induction of RpoS-dependent activities could be separated from those regulated by cyclic AMP (cAMP) or endoinduction, and the induction occurred at extreme glucose limitation. *Escherichia coli* turns to a protective stationary-phase response when nutrient levels fall below approximately 10^{-7} M glucose, which is insufficient to saturate scavenger transporters regulated by cAMP plus endoinducers, and this response is optimally expressed at 10^{-6} M glucose. The high-level induction of protective functions also explains the maintenance energy requirement of bacterial growth at low dilution rates.**

Escherichia coli is thought to be adapted to a feast-and-famine existence (13). However, it could be argued that *E. coli*, at least in the intestine, is rarely subjected to total nutrient deprivation and is more likely to be exposed to low, growth-limiting concentrations of nutrients. Indeed, *E. coli* is extremely adept in competing in an intermediate, nutrient-limited state through upregulation of several high-affinity transport pathways regulated by endogenous inducer synthesis (3, 4, 20). This hunger response contributes to the rapid growth of *E. coli* on micromolar glucose concentrations in steady-state continuous culture. Bacteria adapted in this way have the scavenging capability of oligotrophic bacteria (27).

The pattern of expression of the high-affinity glucose uptake pathway transiently overlaps, at least in nutrient-starved batch culture (3, 20), the patterns of gene expression ascribed to stationary-phase or carbon starvation responses described by other workers (5, 9, 10, 15, 18, 21, 22, 25, 28, 30, 31). Since many of the regulatory changes in stationary phase are actually stimulated by carbon starvation (8, 12, 14) and involve the *rpoS*-encoded sigma factor σ^s, it seems relevant to question whether a particular nutrient threshold induces *E. coli* to optimize persistence and stress resistance rather than continued scavenging and growth. To analyze what growth rate or nutrient concentration triggers the stationary-phase response, this study examined the induction of σ^s-dependent functions in sugar-limited chemostats. As the growth rate in a chemostat is determined by the nutrient concentration in the reaction vessel, chemostats offer a means of finding the threshold concentrations and growth rates triggering physiological responses.

In Fig. 1, we show the dilution rate dependence of three types of regulation in *E. coli* in glucose-limited chemostats. All the bacteria used in comparisons had the same genetic background (strain MC4100) as those used in stationary-phase studies (16). The first type of regulation is typified by the gene *osmY*, which is known to be upregulated by an RpoS-dependent mechanism in stationary phase (16, 32). The periplasmic trehalase (TreA protein) is under similar RpoS control (10). As shown in Fig. 1C and D, the *osmY-lacZ* transcriptional fusion and trehalase activity exhibited parallel patterns of expression under glucose limitation. Induction of *osmY* and TreA activity followed a nearly exponential increase with decreasing dilution rate, with the most drastic increase below a dilution rate of approximately 0.2 h^{-1} and corresponding to a glucose concentration of $<10^{-7}$ M glucose in the chemostat. The extent of induction at a *D* of 0.1 h^{-1} is comparable to the highest levels seen in 24-h stationary-phase batch culture (approximately 115 U of activity for trehalase and 500 U for *osmY-lacZ*). An *rpoS*::Tn*10* mutation greatly reduced expression of *osmY* and *treA* in chemostat cultures (Fig. 1), in confirmation of the role of sigma factor σ^s in regulation of these properties. Also consistent with this view was the finding that Western blots (immunoblots) of *E. coli* extracts produced from bacteria growing at different dilution rates provided qualitative evidence for the presence of RpoS at low dilution rates (D = 0.1 to 0.2 h^{-1}; results not shown).

For comparison with the RpoS-dependent pattern, Fig. 1A and B include two other patterns of expression in response to dilution rate which were previously described (20). The *malT* gene (26) represents an example of gene regulation based solely on cyclic AMP (cAMP) receptor protein-cAMP complex, whose concentration is elevated in glucose-limited chemostats (20). The *lamB* gene encodes a glycoporin involved in glucose scavenging and is regulated by endoinduction as well as cAMP (20). The peak in induction of *lamB* coincides with maximal endogenously produced inducer (maltotriose [24]) levels at a dilution rate of 0.4 to 0.6 h^{-1}, whereas *malT* induction peaks at lower dilution rates in response to cAMP accumulation. As seen from Fig. 1, the pattern of expression of

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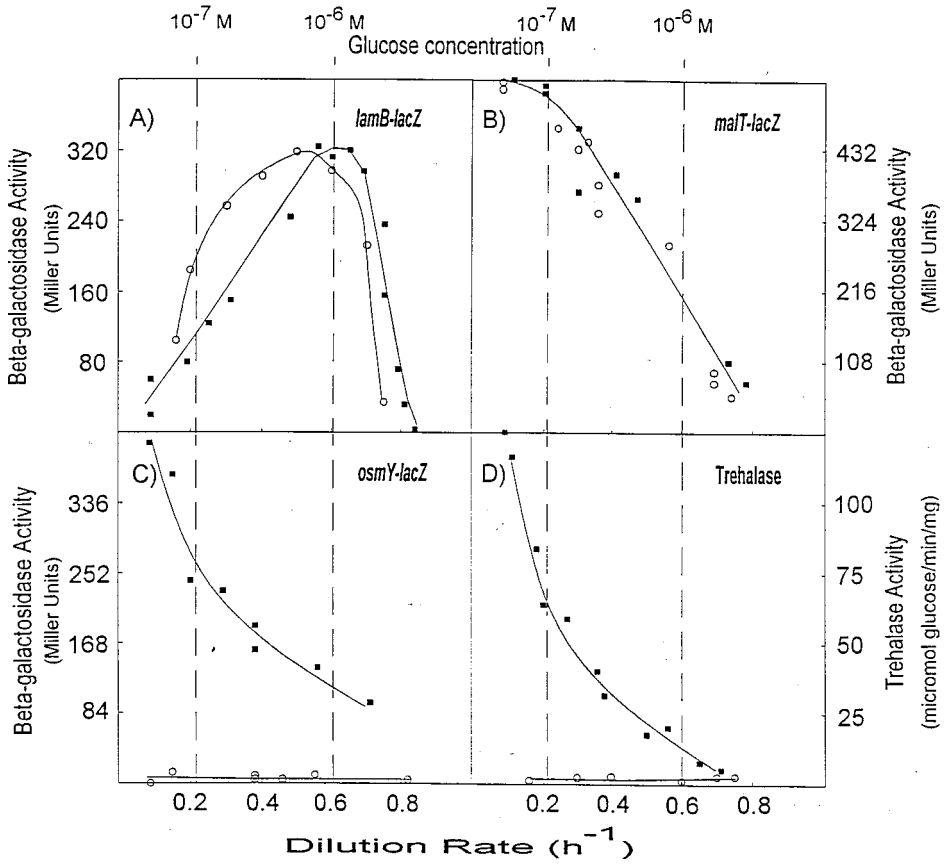

FIG. 1. Induction of genes regulated by cAMP, endoinduction, and the stationary-phase sigma factor σ^s. Induction of activities as a function of growth rate was studied in continuous cultures with bacteria grown in 80-ml glucose-limited chemostats with 0.02% input glucose as previously described (2, 4). Steady-state chemostats were sampled for enzymatic activities at the dilution rates indicated. The glucose concentrations (dashed lines) are based on the published values of Senn et al. (27), whose estimates coincided with the ranges measurable by us at >2 μM (20). (A) The *lamB* gene in glucose-limited chemostats (0.02% feed carbon source) was monitored by measuring β-galactosidase activity of the *lamB-lacZ* fusion in BW2951 (■); this curve is adapted from data from a previous study (20). The same *lamB* fusion in BW2996 (○) (with the *rpoS*::Tn*10* mutation introduced into BW2951 by P1 transduction from ZK1171 [obtained from G. W. Huisman]) was also monitored. (B) The level of *malT* induction in glucose-limited chemostats was determined by assaying the β-galactosidase activity of the *lacZ* fusion in BRE1161 (■), obtained from E. Bremer; this curve is adapted from data from a previous study (20). The effect of an *rpoS*::Tn*10* mutation introduced from ZK1171 into BRE1161 to produce strain BW2950 (○) is also shown. (C) The *osmY-lacZ* fusion activity in strain HYD205 (■) (32) was measured, as well as the same fusion together with an *rpoS*::Tn*10* mutation introduced from ZK1171 into strain BW2953 (○). (D) The periplasmic trehalase activity encoded by *treA* was assayed in BW2951 (■) and BW2996 with the *rpoS*::Tn*10* mutation (○), using an assay based on previously described methods (6) but with the modification that the bacteria were lysed by sonication of a 0.5-ml bacterial suspension concentrated by centrifugation from a 10-ml chemostat sample. The assay, in 1 ml, contained 1 μmol of trehalose as well as 100 μl of 50 mM sodium phosphate buffer (pH 7.5) and 300 μl of bacterial extract in 10 mM phosphate buffer–1 mM $MgSO_4$, pH 7.5. Incubation at 37°C continued for various times before 5 min of boiling to stop the reaction. The glucose produced was assayed with a Sigma glucose oxidase kit according to the manufacturer's procedures, and protein in extracts was assayed with Pearce Chemical's bicinchoninic acid kit.

osmY or *treA* is distinct from that of *lamB* or *malT*, particularly below a *D* of 0.2 h^{-1}. Another obvious difference is that the *rpoS* mutation only marginally shifted the pattern of either *lamB* or *malT* expression in relation to growth rate, so both cAMP and endogenous inducer increase are independent of the function of the sigma factor σ^s.

Trehalose is a compatible solute contributing to osmoprotection and heat resistance (29), and trehalose biosynthesis genes are also under RpoS control (10, 11). As shown in Fig. 2, the intracellular concentration of trehalose rose significantly in response to low dilution rate, as was seen for *osmY* and *treA* expression. The highest concentrations of trehalose accumulated in bacteria at the lowest growth rates tested, corresponding to doubling times of over 7 h. The level of trehalose seen in a culture with a *D* of 0.1 h^{-1} was much higher than the level (below detection) in batch culture stationary-phase bacteria (Fig. 2). The low levels we find in stationary-phase batch culture are consistent with similar findings reported previously (29). It is interesting that more protectant is made at steady-state low growth rates than in the characteristic stationary-phase experiments in batch culture, but the reason is unclear. The *rpoS*::Tn*10* mutation also prevented trehalose formation in chemostat cultures (Fig. 2B), in confirmation of the role of sigma factor σ^s in regulation of trehalose synthesis genes. Hence, the increase in trehalose, as well as of *osmY* and *treA* expression seen at low dilution rates in the *rpoS*$^+$ bacteria, is due to increased RpoS activity at low growth rates.

These results provide new insights into how bacteria regulate their responses to decreasing glucose levels in their environment. It is evident from the comparison in Fig. 1 that scavenging transporters such as LamB are turned on at higher growth rates and higher glucose concentrations than RpoS-dependent functions. Glucose scavenging involves optimized transport of down to 10^{-6} M glucose through the expression of *mgl* and *mal* genes through synthesis of endogenous sugar inducers (2–4, 20). It makes good sense for an organism to turn

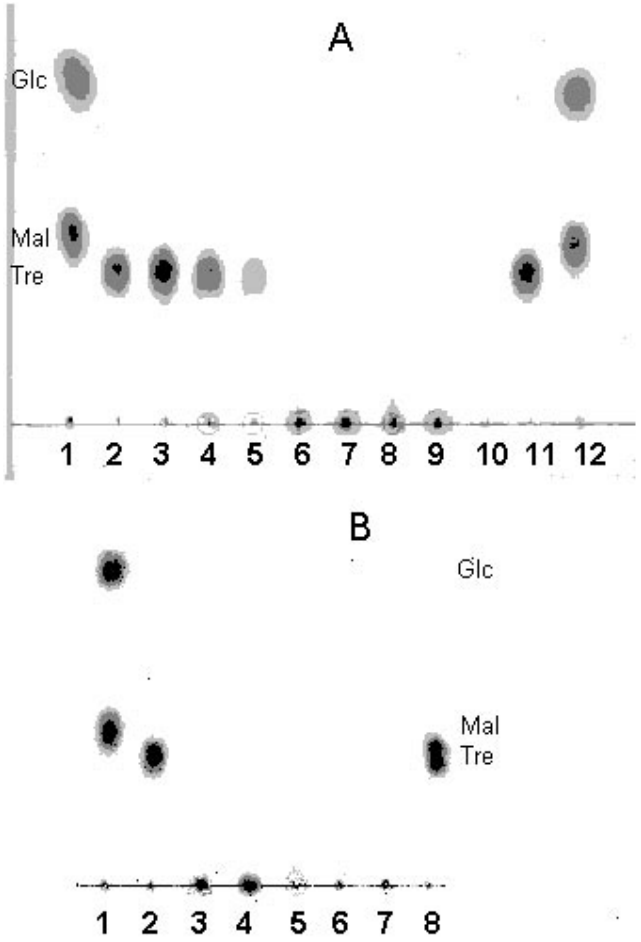

FIG. 2. Detection of trehalose by thin-layer chromatography. BW2951 and BW2996 (*rpoS*::Tn*10*) grown in batch culture and at different dilution rates (4, 20) were extracted from 20-ml culture samples as described previously (1). The extracts were concentrated to 10 μl by freeze-drying, and 4 μl was applied to thin-layer plates and separated as described previously (20). (Plate A) BW2951. Lanes 1 and 12, glucose and maltose standards; lanes 2 and 11, trehalose; lanes 3 to 9, extracts from glucose-limited chemostat cultures grown at dilution rates of 0.09, 0.11, 0.20, 0.3, 0.35, 0.5, and 0.64 h^{-1}, respectively; lane 10, extract from 48-h, stationary-phase batch culture. (Plate B) BW2996. Lane 1, glucose plus maltose; lanes 2 and 8, trehalose standards; lanes 3 to 7, bacterial extracts grown at glucose-limited dilution rates of 0.66, 0.56, 0.39, 0.24, and 0.10 h^{-1}, respectively.

to stationary-phase functions only when the concentration of glucose in the medium is below 10^{-7} M and below the concentration at which even high-affinity transporters are saturated. Indeed, transporter expression switches off coincidentally with the large increase in OsmY, TreA, and trehalose synthesis at low glucose concentrations and with the turning of the bacteria to protective functions.

These results indicate that increased RpoS function does not require an absolute stoppage of growth, and *E. coli* growing on limiting glucose with a doubling time of 7 h or so is well capable of a stationary-phase response. This conclusion is consistent with findings from batch culture starvation experiments, which show that the upturn in RpoS levels starts before the complete stoppage of growth due to starvation (17).

This study is also of relevance in interpreting early chemostat studies of regulatory responses triggered by nutrient limitation, which indicated that different patterns of enzyme induction occurred under carbon-limited growth at different dilution rates (7, 19). Several different patterns of induction of metabolic enzymes were shown in response to changes of growth rate in continuous cultures of *E. coli*. Most of these patterns resemble one of the cAMP, endoinducer, or RpoS-dependent patterns outlined in this study.

Another fundamental feature of bacterial growth is explicable by the massive induction of stationary-phase, protective functions at dilution rates below 0.2 h^{-1}. The extent of trehalose synthesis under carbohydrate limitation must reduce growth yields, although the reduction is difficult to quantitate from the available data. Studies of bacterial growth at low dilution rates have indicated a need for maintenance energy to explain the proportional reduction of growth yields at low growth rates (23). It can be postulated that the energy-intensive synthesis of trehalose and protein due to *rpoS*-controlled functions is a major factor at low dilution rates and contributes to the maintenance energy demands of a slowly growing glucose-limited bacterium.

In summary, bacteria in continuous culture increase RpoS protein levels and RpoS-dependent protective functions characteristic of stationary phase when the medium glucose concentration drops below about 10^{-7} M. The high-affinity glucose uptake pathway involving the LamB glycoporin and the binding-protein-dependent Mgl system is half-saturated at 10^{-6} M (2, 4), so scavenging glucose at 10^{-7} M becomes a drastic problem. *E. coli* clearly regulates an ordered sequence of events at different external nutrient concentrations to optimize bacterial fitness, though the mechanism of sensing of these different nutrient levels is almost entirely unknown.

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