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Growth Phase-Dependent Induction of Stationary-Phase Promoters of *Escherichia coli* in Different Gram-Negative Bacteria

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RSF1010-derived plasmids carrying a fusion of a promoterless *lacZ* gene with the σ^s-dependent growth phase-regulated promoters of *Escherichia coli*, *bolAp*$_1$ and *fic*, were constructed. The plasmids were mobilized into the gram-negative bacterial species *Acetobacter methanolicus*, *Xanthomonas campestris*, *Pseudomonas putida*, and *Rhizobium meliloti*. The β-galactosidase activities of bacterial cultures were determined during exponential and stationary growth phases. Transcriptional activation of the *fic* promoter in the different bacteria was growth phase dependent as in *E. coli* and was initiated generally during the transition to stationary phase. The induction of the *bolA* promoter was also growth phase dependent in the bacteria tested. While the expression in *E. coli* and *R. meliloti* was initiated during the transition from exponential to stationary phase, the induction in *A. methanolicus*, *P. putida*, and *X. campestris* started some hours after stationary growth phase was reached. In all the species tested, DNA fragments hybridizing with the *rpoS* gene of *E. coli* were detected. The results show that in different gram-negative bacteria, stationary-phase-specific sigma factors which are structurally and functionally homologous to σ^s and are able to recognize the promoter sequences of both *bolA* and *fic* exist.

Bacteria have a broad capacity to adapt to changes in the environment. When they are exposed to a variety of environmental stresses or undergo nutrient deprivation, the cells enter stationary phase to cease growth and retain viability for a considerable period. The adaptation to this state requires many physiological changes, including reduction of global gene expression, accompanied by induction of specific genes (14). The synthesis of 30 to 50 proteins is induced in response to starvation. Some of these proteins are induced regardless of the class of nutrient which the cells are starved for (8). A major regulator of the general starvation response has been identified in *Escherichia coli* and is the product of the *rpoS* (*katF*) gene (8, 9, 11). *rpoS* was found to be an RNA polymerase sigma factor (σ^s or σ^{38}) induced early in stationary phase and necessary for transcription of several stationary-phase-specific genes (reviewed in reference 10). Using the purified Eσ^s holoenzyme, Tanaka et al. (16) studied the promoter selectivity in in vitro transcription assays. They found that some promoters are recognized by both Eσ^{70} and Eσ^s holoenzymes whereas others are recognized by one of the two RNA polymerases.

Even though our knowledge is still limited, the data obtained during the last few years indicate that stationary-phase gene regulation in *E. coli* is a complex process involving different regulatory cascades. This might be comparable to differentiation processes found in other bacteria. Until now, few data about the existence of regulatory mechanisms during stationary growth phase, particularly in other bacterial species, have been available. Recently, the *rpoS* genes of *Pseudomonas aeruginosa* and *Salmonella typhimurium* were cloned and analyzed (4, 7).

We initiated an in vivo study to test whether promoters of stationary-phase genes of *E. coli* could be recognized by RNA polymerases of other gram-negative bacteria. For this we chose promoter sequences of the *fic* and *bolA* genes. *fic* is thought to be involved in cell division (5). Its promoter is the only one known to be recognized preferentially by the σ^s cofactor and, apparently, does not require additional transcription factors (16). The gene product of *bolA* is one of the σ^s-dependent regulatory proteins and is involved in morphogenesis (2). Its P1 promoter was shown to be growth phase and growth rate regulated, whereas the upstream P2 promoter is weak and constitutive (1).

To construct vectors suitable for transfer and promoter testing in other gram-negative bacteria, the Ω-Cm cassette of plasmid pHP45Ω-Cm was inserted into the *Eco*RI site of broad-host-range plasmid pRS201 (13). This cassette prevents readthrough of any transcriptionally active sequence located upstream of the multicloning site used for further promoter cloning. The resulting plasmid was designated pGM105 (Table 1). Then, the *Pst*I fragment containing the Km^r gene was replaced by the *Pst*I fragment of pKOK6 (6) containing a *lacZ*-Km^r cassette. The resulting plasmid was designated pGM110 (Table 1). As the promoterless *lacZ* gene is preceded by a ribosome binding site the insertion of promoter sequences upstream of *lacZ* will generate transcriptional fusions. A synthetic DNA region of 48 bp containing the −35 and −10 regions and the transcription start site (+1) of *bolAp*$_1$ (1) was inserted upstream of *lacZ* into pGM110 linearized with *Kpn*I and *Sal*I, resulting in plasmid pGM112 (Fig. 1). The *fic* promoter located on a synthetic 44-bp DNA region was inserted in a similar way except that pGM111, a derivative of pGM110 with a 3-kb *Pvu*II fragment deleted, was used.

As a negative control, pGM118, a derivative of pGM111 from which the DNA region between the Ω cassette and *lacZ* was deleted, was constructed (Table 1). To study the promoter activation of *bolA* and *fic* in *E. coli* and other bacteria, the expression of *lacZ* as a reporter gene was measured by using the β-galactosidase assay (12). When pGM118 was transferred to the *lac* mutant strain *E. coli* DH5α, no β-galactosidase activity was detected, either in exponential or in stationary growth phase. In contrast, when *bolAp*$_1$ or *ficp* was transferred to *E. coli*, expression of β-galactosidase activity was growth phase dependent. The level of β-galactosidase increased rap-

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
E. coli DH5α	*hsdR recA supE* Δ*lacU169*	
E. coli S17-1	*hsdR* Pro *recA*; contains RP4-2-Tc::Mu integrated into the chromosome	15
A. methanolicus MB58(Rf)	Spontaneous mutant of MB58 (17); Rf^r	This work
R. meliloti Rm2011(Sm)	Spontaneous mutant of Rm2011; Sm^r	U. Priefer, University of Bielefeld
P. putida DSM291(Rf)	Spontaneous mutant of DSM291; Rf^r	This work
X. campestris pv. *campestris* B100	Sm^r	D. Steinmann, University of Bielefeld
Plasmids		
pRS201	Derivative of RSF1010 containing a Km^r gene and a multicloning site; Km^r	13
pHP45Ω-Cm	Contains an interposon with the Cm^r gene from pKT210; Ap^r Cm^r	3
pKOK6	Contains a *lacZ*-Km^r cassette	6
pGM105	Derivative of pRS201 containing the interposon from pHP45Ω-Cm	This work
pGM110	Derivative of pGM105; the *Pst*I fragment containing Km^r was replaced by the *lacZ*-Km^r cassette of pKOK6	This work
pGM111	Derivative of pGM110 with a 3-kb *Pvu*II fragment deleted	This work
pGM112	Derivative of pGM110 containing the promoter region of *bolA* of *E. coli*	This work
pGM115	Derivative of pGM111 containing the promoter region of *fic* of *E. coli*	This work
pGM118	Derivative of pGM111; DNA region between Ω cassette and *lacZ* deleted	This work
pRH320	Derivative of pBR322 containing a 4.35-kb insert with the *rpoS* gene of *E. coli* K-12	8

idly at the beginning of the transition to stationary phase. When the maximal cell number was reached, enzyme activity increased further at a lower rate during the following 24 h. The amount of β-galactosidase activity induced by the *fic* promoter was approximately 20% of that induced by $bolAp_1$. Both the level of expression and the activation pattern of the promoters corresponded with the results obtained by other authors for chromosomal *fic-lacZ* and $bolAp_1$-*lacZ* fusions (5, 9). Moreover, although the −35 region of *fic* is not yet defined, the data indicate that the DNA sequence of the synthetic DNA region cloned on pGM115 contained all the genetic information necessary for its correct recognition and growth phase-regulated transcription.

To study the expression of $bolAp_1$ and *ficp* in other gram-negative bacteria, pGM112 and pGM115, respectively, were transferred to *Acetobacter methanolicus*, *Rhizobium meliloti*, *Pseudomonas putida*, and *Xanthomonas campestris*. We were interested in involving different genera in the experimental program. For selection of transconjugants, spontaneous resistance mutants of wild-type strains were used. In addition, all recipients were *lacZ* mutants, as shown on agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and confirmed by β-galactosidase assay (<1 Miller unit; *P. putida*, *A. methanolicus*, and *X. campestris*). Only *R. meliloti* 2011 had a weak natural β-galactosidase activity (approximately 20 Miller units). As both plasmids pGM112 and pGM115 are derivatives of the broad-host-range plasmid RSF1010, they were not self-transmissible but could be mobilized easily from *E. coli* S17-1 (15).

As shown in Fig. 2, in all bacterial species tested the expression of the *fic* promoter was strongly growth phase dependent and was initiated generally during the transition to stationary phase, like in *E. coli*. But, there are differences between the expression levels in the bacteria. The maximal level of β-galactosidase activity ranged from 50 Miller units for *E. coli* to 1,800 and 2,450 Miller units for *R. meliloti* and *A. methanolicus*, respectively. Despite these differences, the activation rate (ratio [Miller units] of stationary phase to exponential phase) was comparable and ranged from 5 (*E. coli*) to 12 (*P. putida*). These results suggest that a putative stationary-phase-specific RNA

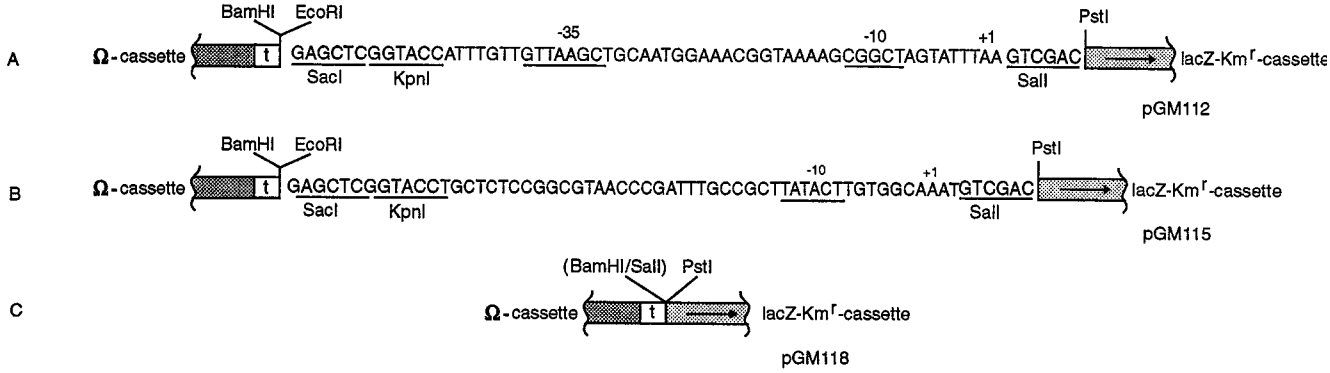

FIG. 1. Insertion sites of the synthetic promoter regions of *bolA* (A) and *fic* (B) in pGM112 and pGM115, respectively. The nucleotide sequence is given in the 5′-to-3′ direction. The corresponding structure of pGM118 (negative control without promoter) is also presented (C). t, T4 transcription termination signal of the Ω-Cm cassette.

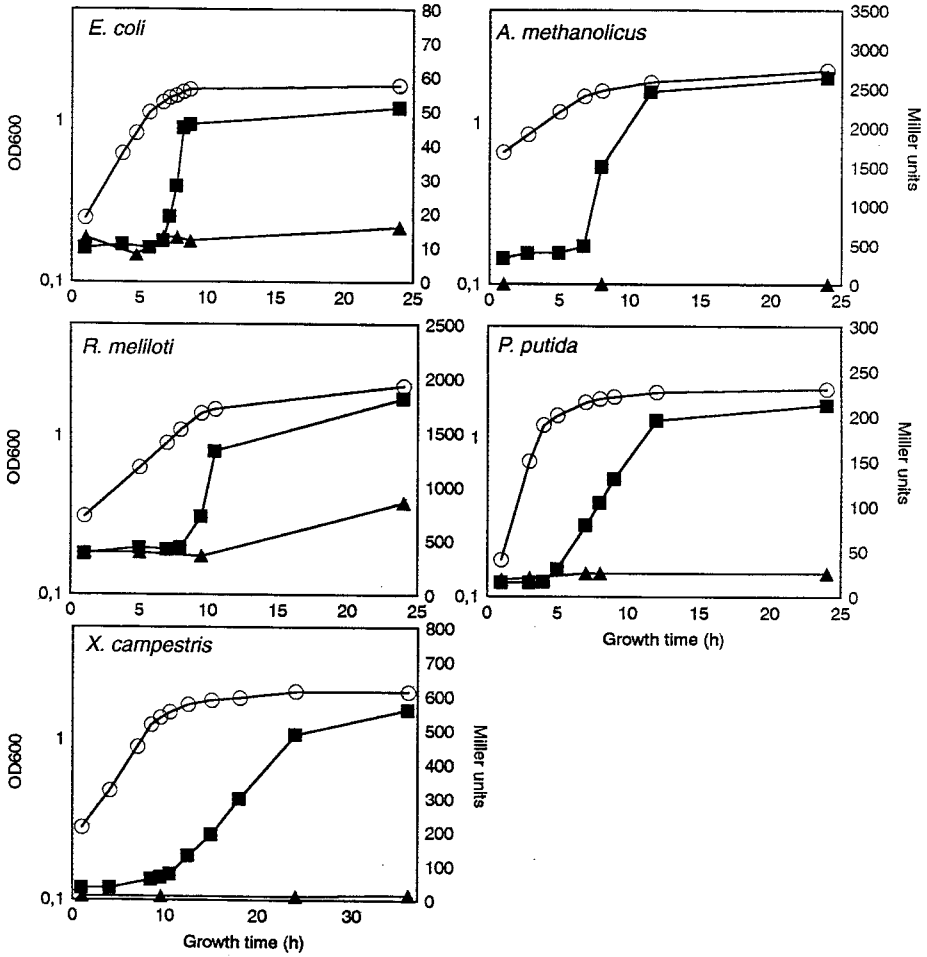

FIG. 2. Growth phase-dependent induction of *lacZ* by the *fic* promoter (pGM115) in *E. coli*, *A. methanolicus*, *R. meliloti*, *P. putida*, and *X. campestris*. Cells were grown in complete medium (Luria-Bertani medium). The cultures were inoculated with bacteria in exponential growth phase. The optical density at 600 nm (OD600) was monitored (circles), and specific β-galactosidase activity was determined (rectangles). As a negative control (triangles), plasmid pGM118 was used.

polymerase with functional homology to $E\sigma^{38}$ of *E. coli* exists in the strains investigated. This holoenzyme can correctly recognize the growth phase-regulated *fic* promoter sequence of *E. coli*. Since the DNA sequence is within a fragment of only 44 bp, the identical promoter consensus sequence is apparently recognized in different bacteria.

The results of $bolAp_1$-mediated expression of *lacZ* in different bacteria are summarized in Fig. 3. In all bacteria tested, the expression of β-galactosidase was strongly growth phase dependent and was induced during stationary growth phase. The level of expression differed considerably between species. For instance, the maximal level in *R. meliloti* was almost 10-fold higher than in other strains. On the other hand, the induction ratio of $bolAp_1$ was similar in all strains (ranging from 6 to 9).

In contrast to the results obtained for the *fic* promoter, for which activation usually started during the transition to stationary phase, the increase of β-galactosidase activity was clearly delayed for $bolAp_1$ in *A. methanolicus* and *P. putida*. In these strains the enzyme activity increased only after cells had been in stationary phase for some hours. An extremely long cultivation time was necessary for promoter activation in *X. campestris*. Among different bacterial species, not only does the time of induction vary between *fic* and *bolA*, but the levels of expression vary as well. In *X. campestris*, *A. methanolicus*, and *P. putida*, the level of stationary-phase expression of *fic* is up to 50-fold (*A. methanolicus*) higher than in *E. coli* but *bolA* expression is similar or even lower (*X. campestris*). This suggests a differential control of *fic* and *bolA*, in addition to a putative common dependence on σ^s, which is different in the various species. Since the *lacZ* mRNA molecule generated by transcription of $bolAp_1$ is almost identical to that of the *fic* promoter, differences in β-galactosidase expression of cells containing pGM112 or pGM115 reflect differences in transcription initiation rather than differences in mRNA stability or translation initiation. Our results indicated that both promoters were recognized in different gram-negative bacteria by a stationary-phase-specific RNA polymerase.

To test whether sequences with structural homology to *rpoS* of *E. coli* exist in the other gram-negative bacteria used, genomic DNA digested completely with *Eco*RI (*A. methanolicus* and *R. meliloti*), *Pst*I (*P. putida* and *X. campestris*), or *Cla*I (*E. coli*) was hybridized with pRH320 containing a 4.35-kb *Cla*I insert with the *rpoS* gene of *E. coli* (8) (Table 1). For all strains tested, Southern blotting and nonradioactive labeling with the digoxigenin system yielded a hybridization signal with the probe (pRH320). In *P. putida* and *X. campestris* a 3.8- and a 3.9-kb *Pst*I fragment, respectively, hybridized with the *E. coli* probe, and in *A. methanolicus* and *R. meliloti* a 3.3- and a 3.8-kb

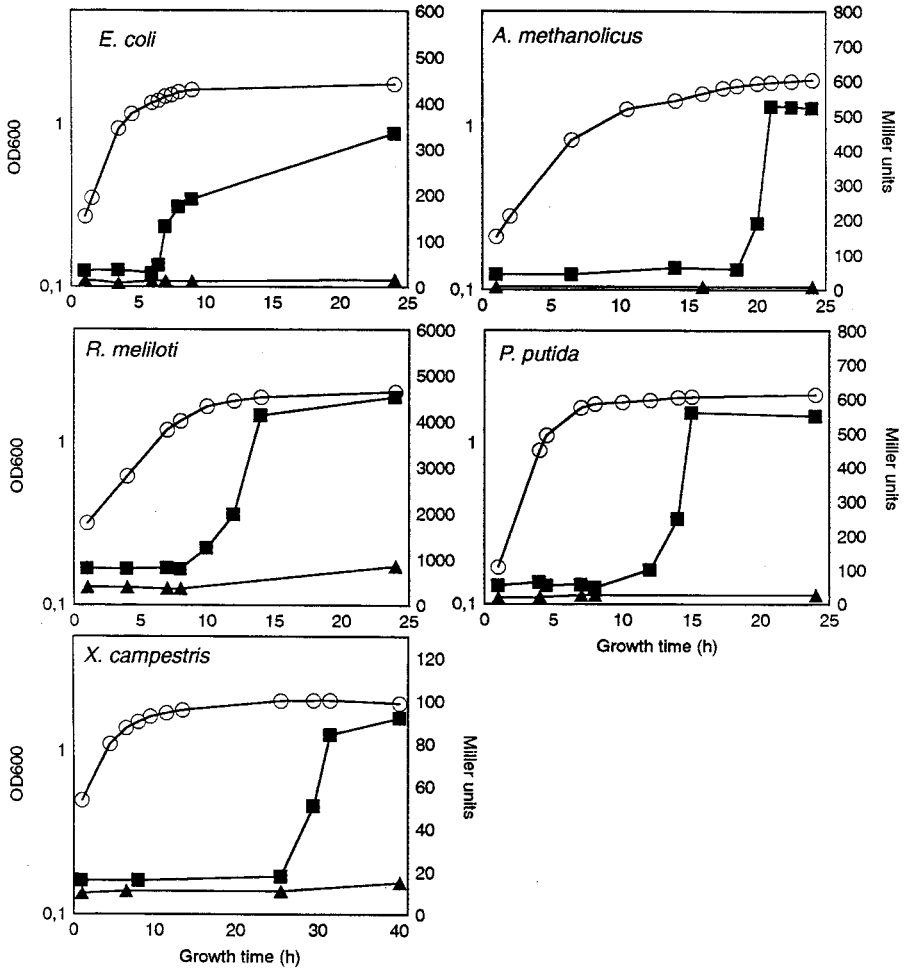

FIG. 3. Growth phase-dependent induction of *lacZ* by *bolAp*$_1$ (pGM112) in *E. coli*, *A. methanolicus*, *R. meliloti*, *P. putida*, and *X. campestris*. Culture conditions, symbols, and abbreviations are as described in the legend to Fig. 2.

*Eco*RI fragment, respectively, hybridized with the probe (Fig. 4). As expected, in *E. coli* a 4.3-kb *Cla*I fragment hybridized with pRH320 containing the same fragment. This indicates that in other gram-negative bacteria there are DNA sequences homologous to *rpoS* of *E. coli*. As the hybridization signal was much stronger in *E. coli* than in the other species, we suggest that the homology between *rpoS* of *E. coli* and analogous genes of the other bacteria is relatively weak. An extremely weak hybridization band was obtained with *A. methanolicus*, indicating a very low level of homology.

According to their relationship in the phylogenetic tree as determined by 16S rRNA sequences (18), all bacterial species used here belong to the division of purple bacteria containing most, but not all, of the traditional gram-negative bacteria. *E. coli*, *P. putida*, and *X. campestris* are members of subdivision γ, whereas *R. meliloti* belongs to subdivision α. The position of *A. methanolicus* within the division of purple bacteria has not been tested until now. Our results show that bacterial species with different phylogenetic distances within the division of purple bacteria have a growth phase-dependent gene expression similar to that in *E. coli.*

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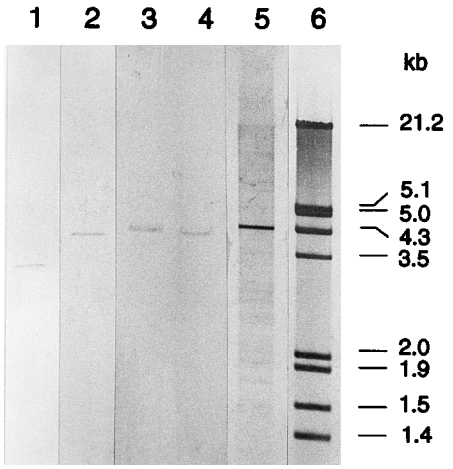

FIG. 4. Southern blot hybridization performed on total DNA prepared from several gram-negative bacterial strains. Agarose gel electrophoresis and Southern transfer were followed by hybridization to digoxigenin-labeled pRH320. Lane 1, *A. methanolicus* digested with *Eco*RI; lane 2, *P. putida* digested with *Pst*I; lane 3, *X. campestris* digested with *Pst*I; lane 4, *R. meliloti* digested with *Eco*RI; lane 5, *E. coli* (MC4100) digested with *Cla*I; lane 6, λ marker III (Boehringer).

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