

## Penicillin-Binding Proteins Are Regulated by *rpoS* during Transitions in Growth States of *Escherichia coli*

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Attention has been recently focused on the role of the *rpoS* (formerly *katF*) gene product as a regulator during the transition from the exponential growth phase to the stationary phase as well as during nutritional starvation. It has been demonstrated that RpoS is an alternate sigma factor which would bind to promoters of genes induced at these times. It was previously noted that *rpoS* mutants do not undergo a transition to short rods during entry into the stationary phase. Because of their well-established role in morphogenesis, we investigated the status of the penicillin-binding proteins (PBPs) in *Escherichia coli* wild-type and isogenic *rpoS* mutants. Samples from cultures of *E. coli* ZK126 and ZK1000 (*rpoS*::kan) were taken in the midlogarithmic, early stationary, and late (24 h) stationary phases. The increase in PBP 6 seen upon entry of the wild-type strain into the stationary phase was not observed with the *rpoS*::kan cells, even after 24 h. There was also a marked decrease of PBP 3 in wild-type stationary-phase cells; PBP 3 has a known influence on morphogenesis. This decrease in PBP 3 was found to be markedly affected by the disruption of *rpoS*. Similar observations were made after prolonged starvation of the two strains for either glucose or a required amino acid. Inasmuch as PBPs are involved in peptidoglycan synthesis, we also examined two properties of peptidoglycan, autolysis and cross-linkage, that might be altered by the PBP differences. However, neither of these properties, which are known to undergo changes in the stationary phase, appeared to be influenced by the status of RpoS.

Recent work has revealed that the transition of *Escherichia coli* from the logarithmic phase of growth into the stationary phase is not a passive process; rather, the expression levels of a number of gene products undergo substantial changes during this period. The regulatory mechanisms are believed to have evolved because many bacteria encounter in their environments circumstances in which nutrients are limited (12). Starvation or entry into the stationary phase also leads to the development in cells of resistance to a broad range of environmental insults or stresses (12, 20, 21, 27). The *rpoS* (formerly *katF*) gene (16) has been implicated as a major control element in this process, and evidence that *rpoS* is an alternate sigma factor ( $\sigma^{38}$ ) for RNA polymerase has been presented (22, 31). The factors that act to induce RpoS are still under investigation (12, 23, 26). The *rpoS* gene is apparently essential for continued cell survival during prolonged periods in the stationary phase (15). A number of genes that have changed expression levels upon entry into the stationary phase have been shown to be RpoS dependent; these include catalase genes such as *katE*, exonuclease III (*xthA*), the *osmB* lipoprotein, and acidic phosphatase (*appA*), as well as others (5, 12, 14, 15, 19, 25, 32). Also among the RpoS-regulated genes is *bolA*, which encodes a small regulatory protein involved in the control of several genes, including penicillin-binding protein (PBP) 6 expression (1, 2). In addition, wild-type cells tend to have short coccobacillary forms in the stationary phase, whereas *rpoS*-disrupted strains have heterogeneous cell lengths (17). These observations suggest that the RpoS protein might exert regulatory influences on components of the cell wall synthesis apparatus.

It is known that bacteria in either the stationary phase or

nongrowing bacteria are not susceptible to the killing and lytic effects of  $\beta$ -lactam antibiotics, a phenomenon termed "phenotypic tolerance" (11). In the present study, the contribution of the *rpoS* gene to the regulation of the targets for and effects of  $\beta$ -lactam antibiotics was assessed. PBPs are the enzymes that catalyze the terminal stages of peptidoglycan assembly (30). In addition, PBPs have defined morphogenic roles in cell growth and division (29). It was previously demonstrated that there are several differences in PBP expression levels between logarithmic- and stationary-phase *E. coli* (3). To date, however, there has been no systematic study of the possible regulatory role of RpoS on PBP expression and its potential role in phenotypic tolerance.

In the present experiments, the effect of an *rpoS* disruption (*rpoS*::kan) on the expression of the PBPs during the logarithmic and stationary phases was determined. During the stationary phase, in addition to the expected RpoS dependence of the upregulation of PBP 6 through BolA, we also observed that the downregulation of PBP 3 was affected by the disruption of *rpoS*. Virtually identical effects were obtained by two different nutritional starvation protocols. The disruption of *rpoS* did not, however, have an influence on ampicillin- or imipenem-induced killing of nongrowing cells, i.e., phenotypic tolerance. Cell wall characteristics that change during the shift from the logarithmic to the stationary phase, such as autolysis induction and peptidoglycan cross-linkage, were also studied. In contrast to the effects observed on the PBPs, these two parameters did not appear to be influenced by the disruption of the *rpoS* gene.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *E. coli* MC4100 was from the laboratory collection; *E. coli* ZK126 (W3110  $\Delta$ *lacU169 tna-2*), and the isogenic strain ZK1000 (ZK126 *rpoS*::kan) were obtained from D. Bohannon (2). *E. coli* UM122 (HfrH, *thi-1 rpoS*::Tn10) was provided by P. Loewen

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(23), and *E. coli* KL334 (*lacI22*, *lacZ118*,  $\lambda^-$ , *lysA23*) was from B. Bachmann (*E. coli* Genetic Stock Center, Yale University, New Haven, Conn.). Transductions were carried out by using the P1 *vir* phage as described previously (28). Turbidity was monitored at 610 nm on a Sequoia-Turner model 340 spectrophotometer. For experiments with stationary-phase bacteria, strains were grown overnight in tryptone broth (28) at 30°C, and the next morning a 0.1% inoculum was placed into 600 ml of Lennox L broth (LB; GIBCO BRL, Gaithersburg, Md.). Growth was continued at 37°C in LB with vigorous aeration. Starvation experiments were carried out with M9 medium (28) supplemented with 0.2% glucose–0.1  $\mu$ g of thiamine per ml–50  $\mu$ g of lysine per ml. Cells were grown overnight at 37°C in M9 medium with supplements, and a 0.5% inoculum was placed into 600 ml of prewarmed M9 medium with supplements. The effect of glucose or amino acid deprivation on PBP expression was determined as follows. Either strains ZK126 and ZK1000 (glucose starvation) or strains KL334 and KL334 *rpoS::kan* (lysine starvation) were grown in supplemented M9 medium. Starvation was initiated at an  $A_{610}$  of 0.4 to 0.5 by harvesting the cells by centrifugation (5,000  $\times$  *g*, 10 min), washing them in one volume of prewarmed M9 medium lacking either glucose or lysine, and resuspending the cells in prewarmed M9 medium lacking the desired supplement. The final resuspension was designated time zero for subsequent measurements.

**P1 transduction of *rpoS::kan*.** In order to label the peptidoglycan with [<sup>3</sup>H]diaminopimelic acid, it was necessary to construct a *lysA rpoS*-disrupted strain (34). P1 *vir* phage was propagated on *E. coli* ZK1000, and the lysate was used to infect *E. coli* KL334 by standard methods (28). After a 60-min incubation period, cells were plated on LB agar (GIBCO BRL) with 20  $\mu$ g of kanamycin per ml to select for *rpoS::kan* transductants. The transduction of the *rpoS* disruption was confirmed by streaking single kanamycin-resistant transductants onto LB-kanamycin agar plates and testing some of the resultant colonies with a drop of hydrogen peroxide. An *rpoS::tet* strain of *E. coli* MC4100 was constructed in an analogous manner by using *E. coli* UM122 to propagate the P1 transducing phage.

**PBPs.** Cells (ca. 300 ml in the logarithmic phase or starvation protocols; 100 ml in stationary phase) were harvested, chilled in an ice-water bath, and collected by centrifugation at 7,000  $\times$  *g* for 10 min at 4°C. The cells were washed with 100 ml of ice-cold sodium phosphate buffer (10 mM; pH 7.0) and were pelleted as described above. The pellet was rapidly frozen in dry ice-alcohol and was kept at –70°C overnight. Membranes were prepared from the thawed pellets by sonication and differential centrifugation as described previously (24, 29). After determining the protein concentration, membranes were subsequently suspended in 0.5 ml of phosphate buffer, and the mixture was frozen at –70°C until used in the PBP assay. The PBP assay was carried out essentially as described previously (29) with [<sup>3</sup>H]benzylpenicillin (26 Ci/mmol; DuPont NEN, Boston, Mass.). In order to increase the resolution, gels were run on a long bed (20 cm) at a constant current of 10 mA at 4°C for 16 h. Quantitative fluorography and scanning with an LKB laser densitometer were performed as described previously (24).

**Antibiotic-induced kill rates.** The abilities of ampicillin (Sigma Chemical, St. Louis, Mo.) and imipenem (Merck & Co., Rahway, N.J.) to kill nongrowing wild-type and *rpoS::kan* strains were measured. Loss of viability was determined after 3 h of antibiotic exposure. Samples of 0.1 ml were removed, serial 10-fold dilutions were made in phosphate-buffered saline (pH 7.2), and 0.1 ml of the appropriate dilutions was plated in triplicate onto LB agar plates. The CFU was determined after

18 h of incubation at 37°C. Experiments in which killing was measured in lysine-starved cells used KL334 strains and were initiated by collecting on a sterile membrane filter 30 ml of logarithmic-phase cells grown in M9 medium with glucose and lysine. The cells were washed twice by filtration with prewarmed M9 medium with glucose but lacking lysine and were subsequently resuspended in warm M9 medium with glucose. The total time for this operation was less than 5 min.

**Autolysis induction.** Induction of autolysis was carried out essentially as reported previously (18) by using 0.1 M Tris-Cl (pH 8.0) and 0.2 mM EDTA. Rates were measured at 610 nm with a Sequoia-Turner model 340 spectrophotometer at 37°C.

**Peptidoglycan preparation and analysis.** *E. coli* KL334 (*lysA*) and the isogenic KL334 (*lysA rpoS::kan*) strain constructed by transduction were used to label peptidoglycan with [<sup>3</sup>H]diaminopimelic acid (23 Ci/mmol; Research Products International Corp., Mount Prospect, Ill.). A 50-ml culture in LB with 50  $\mu$ g of lysine per ml was grown at 37°C with aeration. At the midlogarithmic phase and the early stationary phase, 10 ml of cells was removed and placed in a flask with 30  $\mu$ Ci of [<sup>3</sup>H]diaminopimelate, and the cells were grown for 30 min with the label. Unlabelled diaminopimelate was then added, and incubation was continued for 30 min prior to harvesting. In the case of the overnight time point, the cells were incubated in the early stationary phase for 30 min with the labelled diaminopimelate because of the low rate of incorporation in late-stationary-phase cells. Subsequently, unlabelled diaminopimelate was added, with incubation continued overnight until harvesting at 24 h. In each case, the cells were then rapidly chilled on ice, collected by centrifugation, and resuspended in ice-cold sodium acetate buffer (50 mM; pH 5.5) and were then added dropwise to an equal volume of boiling 8% sodium dodecyl sulfate (SDS) (9). After 30 min of boiling, approximately 10 mg of unlabelled carrier peptidoglycan was added, and the peptidoglycan fraction was collected by centrifugation at 150,000  $\times$  *g* for 30 min. The peptidoglycan was subsequently washed three times with distilled water and was collected by centrifugation at 150,000  $\times$  *g* as described above. After digestion with trypsin for 60 min (200  $\mu$ g in 2 ml of 50 mM Tris-Cl [pH 8.0] with 10 mM CaCl<sub>2</sub>), the peptidoglycan was again boiled in 0.1% SDS and was washed four times as described above (4, 7). The material was digested with 20  $\mu$ g of *N*-acetylmuramidase SG (United States Biochemicals, Cleveland, Ohio) in 100  $\mu$ l of acetate buffer (pH 5.5) for 16 h, and then ca. 100,000 dpm was spotted onto plastic-backed silica gel thin-layer chromatography plates (Eastman Kodak, Rochester, N.Y.). The plates were developed with isobutyric acid–1 M NH<sub>4</sub>OH (5:3) as described previously (7, 10). After spraying with En<sup>3</sup>Hance (DuPont NEN), the chromatographs were exposed to Kodak X-Omat AR film (Eastman Kodak) at –70°C for 48 h. The resulting films were used as templates to locate and cut out the radioactive regions representing the muropeptide species from the plastic-backed chromatograms (10). These were transferred to scintillation vials, and after scintillation counting (Beckman LS5000TD), cross-linkage was determined by the formula of Driehuis et al. (8).

## RESULTS

**PBP effects.** The isogenic strains *E. coli* ZK126 and ZK1000 (*rpoS::kan*) were grown in LB and samples were taken at the midlogarithmic, stationary (defined as 2 h after the initial decrease in the growth rate as measured by optical absorbance), and overnight (24 h after initial inoculation) time points. The culture samples were sonicated, the membranes were prepared, and [<sup>3</sup>H]penicillin was bound at saturating

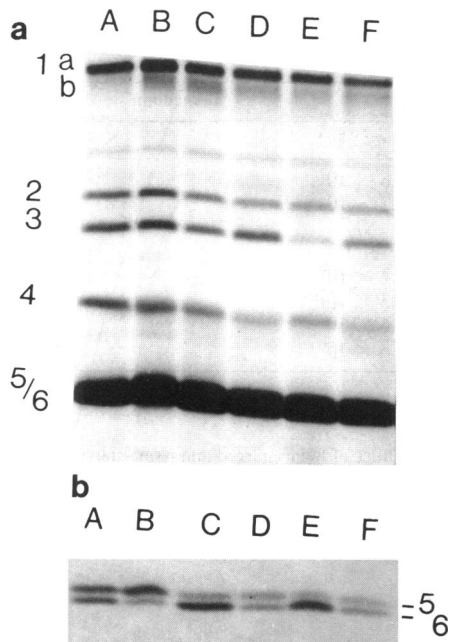


FIG. 1. PBP patterns of *E. coli* of ZK126 and ZK1000 (*rpoS::kan*) during the logarithmic phase, stationary phase, and 24 h (overnight) of culture. The PBPs of *rpoS*<sup>+</sup> (lanes A) and *rpoS::kan* (lanes B) in the logarithmic phase, *rpoS*<sup>+</sup> (lanes C) and *rpoS::kan* (lanes D) in the early stationary phase, and *rpoS*<sup>+</sup> (lanes E) and *rpoS::kan* (lanes F) after overnight culture are illustrated. Note the difference in the PBP 3 levels in panel a. The PBP 5/6 group is overexposed in the fluorograph in panel a and is illustrated with a shorter exposure in panel b to demonstrate the PBP 6 differences. See Table 1 for quantitation by laser densitometry.

concentrations. Subsequently, gel electrophoresis and fluorography were performed. The results of the PBP assay showed that the previously reported changes (3) in PBPs 3 and 6 during the stationary phase, reproduced in ZK126 here in the present study, were substantially altered in the *rpoS::kan* strain. As illustrated in Fig. 1 and Table 1, the decline in the amount of PBP 3 relative to the decline in the amount of other PBPs seen as cells entered the stationary phase was substantially less in the *rpoS*-disrupted strain. Similar measurements also showed that the increase in PBP 6 observed in the wild-type *rpoS* strain was absent from the isogenic *rpoS*-disrupted strain (Table 1). It should be noted that the *rpoS::kan* strain consistently had a decreased level of PBP 6 in the logarithmic phase of growth (Fig. 1) compared with that of the parent strain, and this discrepancy increased further in the stationary phase. There was also a modest effect of *rpoS* status on PBP 4 in the overnight stationary phase, in that the amount of PBP 4 in the

TABLE 1. PBP ratios as a function of growth phase

Sample time <sup>a</sup>	<i>rpoS::kan/rpoS</i> <sup>+</sup> PBP 3 ratio <sup>b</sup>	<i>rpoS::kan/rpoS</i> <sup>+</sup> PBP 6 ratio <sup>b</sup>
Logarithmic phase	0.94	0.55
Stationary phase	1.65	0.36
Overnight (24 h)	3.77	0.28

<sup>a</sup> Samples were taken at the indicated points, as defined in the text.

<sup>b</sup> Expressed as a ratio of the PBP content of the *rpoS*-disrupted strain over that of the wild-type strain. PBP quantities were calculated from areas under the curve derived by laser densitometric scanning of the fluorograph. Results are average values of three independent determinations.

TABLE 2. Effect of starvation on PBP ratio

Starvation	Time of sampling <sup>a</sup>	<i>rpoS::tet/rpoS</i> <sup>+</sup> PBP 3 ratio <sup>b</sup>	<i>rpoS::tet/rpoS</i> <sup>+</sup> PBP 6 ratio <sup>b</sup>
Glucose	Logarithmic	1.03	0.56
	Starved for 2 h	1.13	0.42
	Starved for 18 h	5.0	0.47
Lysine	Logarithmic	0.94	0.73
	Starved for 2 h	0.85	0.58
	Starved for 18 h	>5.0 <sup>c</sup>	0.45

<sup>a</sup> Logarithmic indicates the logarithmic phase just prior to the beginning of starvation.

<sup>b</sup> Ratios of PBPs calculated as described in footnote b of Table 1. Results are averages of three determinations.

<sup>c</sup> The wild-type levels of PBP 3 band density were below the levels that could accurately be detected with the densitometer.

overnight *rpoS*-disrupted strain was about 60 to 70% of the value for the wild-type strain. Another isogenic pair, *E. coli* MC4100 and MC4100 *rpoS::tet*, exhibited PBP effects identical to those described above (data not shown).

The effects of glucose deprivation and removal of an essential amino acid were examined. Either ZK126 and ZK1000 (glucose starvation) or KL334 and KL334 *rpoS::kan* (lysine starvation) were used. The results of the experiments are tabulated in Table 2. Both starvation protocols had similar effects on PBP 3, in that prolonged (18 h) deprivation of either glucose or lysine resulted in markedly decreased levels of PBP 3 in the isogenic parent strain relative to those in the *rpoS*-disrupted strain. Short exposure times (2 h) had a minimal if any effect. The differences in the levels of PBP 6 for cells with short exposure times were more modest than those for cells in the stationary phase in the case of both lysine deprivation and glucose starvation.

**Antibiotic-induced killing.** To determine whether the differences in PBP levels that we observed (see above) contributed to the phenomenon of phenotypic tolerance, ampicillin-induced cell killing was measured in parental and *rpoS*-disrupted strains. A 3-h antibiotic treatment was selected to ensure adequate exposure times. As can be seen in Table 3, the viabilities of both parental and *rpoS*-disrupted strains decreased approximately 1,000-fold during logarithmic-phase exposure to ampicillin. However, neither isogenic strain showed any measurable decline in viability when exposed to ampicillin after being in the stationary phase or upon amino acid starvation for 2 h. With imipenem, which has been shown to kill *E. coli* for a period following the start of amino acid starvation (33), a 10-fold decrease in viability over 3 h was observed when the antibiotic was added 10 min after the start of amino acid deprivation. There did not, however, appear to be a significant difference in killing between the intact and *rpoS*-disrupted strains.

**Cell wall effects.** Autolysis rates were measured by using cells that had been rapidly centrifuged and resuspended in 0.1 M Tris-Cl (pH 8.0)-0.2 mM EDTA (18). After a reading at time zero, the cells were placed at 37°C, and the absorbance was measured at 5-min intervals. As seen in Fig. 2A, logarithmic-phase cells of both the parent strain and the *rpoS::kan* strain autolysed rapidly and at the same rates when suspended in alkaline Tris buffer with EDTA. In contrast, cells from the early stationary phase (Fig. 2B) and after 24 h in culture (Fig. 2C) autolysed much less rapidly when they were resuspended in buffer. The degree of autolysis and the rate of autolysis appeared to be independent of the functional status of the *rpoS* gene.

TABLE 3. Effect of growth status on cell viability after ampicillin treatment

Growth status	CFU/ml			
	LB		M9 medium	
	<i>rpoS</i> <sup>+</sup>	<i>rpoS</i> ::kan	<i>rpoS</i> <sup>+</sup>	<i>rpoS</i> ::kan
Logarithmic	$5.8 \times 10^8$	$3.9 \times 10^8$	$6.8 \times 10^7$	$1.1 \times 10^8$
+ Ampicillin <sup>a</sup>	$3.2 \times 10^4$	$1.7 \times 10^5$	$3.2 \times 10^4$	$1.7 \times 10^5$
Stationary	$3.1 \times 10^9$	$5.1 \times 10^9$	ND <sup>b</sup>	ND
+ Ampicillin	$3.0 \times 10^9$	$6.2 \times 10^9$	ND	ND
Lysine-starved <sup>c</sup>	ND	ND	$8.4 \times 10^7$	$1.0 \times 10^8$
+ Ampicillin	ND	ND	$8.3 \times 10^7$	$1.2 \times 10^8$
Lysine-starved	ND	ND	$4.6 \times 10^7$	$1.1 \times 10^8$
+ Imipenem <sup>d</sup>	ND	ND	$3.8 \times 10^6$	$5.8 \times 10^6$

<sup>a</sup> Ampicillin was present for 3 h at 20  $\mu$ g/ml (50 $\times$  the MIC).

<sup>b</sup> ND, not determined.

<sup>c</sup> Lysine starvation for 2 h.

<sup>d</sup> Imipenem (3  $\mu$ g/ml; 10 $\times$  the MIC) was added 10 min after the start of lysine deprivation. The viabilities of lysine-starved and lysine-starved, imipenem-treated cultures were measured after 3 h of antibiotic exposure.

Another cell wall property known to undergo changes during the transition from the logarithmic to stationary phase, peptidoglycan cross-linkage, was also compared in the wild-type and *rpoS*::kan strains. In order to radiolabel the peptidoglycan, the *rpoS*::kan construct was transduced via P1 phage into KL334, a *lysA* strain (34). The isogenic *lysA* and *lysA rpoS*::kan strains were grown in LB broth–50  $\mu$ g of lysine per ml and were labeled with [<sup>3</sup>H]diaminopimelic acid during the logarithmic phase (30-min label), the early stationary phase (30-min label), and the early stationary phase (30-min label) with subsequent overnight incubation. In each case, the peptidoglycan was prepared and digested with trypsin to remove bound lipoprotein and subsequently with *N*-acetylmuramidase SG to distinguish the muropeptides (4, 9, 10). Analysis was by thin-layer chromatography on silica gel with fluorography to locate the radioactive muropeptides. The results are tabulated in Table 4. As expected (8), the cross-linkage index for the cells increased after an extended stationary phase; however, no significant differences between the parental and *rpoS*::kan cells at the various stages were noted. The cross-linkage values were in close agreement with those reported by other workers (8).

## DISCUSSION

The results of the present study strongly suggest that the *rpoS* gene has an influence on the changes in the PBP pattern observed when *E. coli* enters the stationary phase or during prolonged nutrient starvation. The observed change in PBP 6 would be predicted on the basis of earlier work by Lange and Hengge-Aronis (17) and Aldea (1). In the former study, the growth phase-regulated expression of *bolA* was shown to be controlled by RpoS, while the latter study demonstrated that PBP 6 expression could be modulated by BolA. The finding reported here that PBP 3 is also influenced by RpoS prompted us to look upstream in the published *ftsI* sequence for the proposed tentative consensus sequences for *rpoS*-regulated promoters (17). No obvious good match was found in a region extending 620 bp upstream, either by inspection or by a computer-based search with the GenePro software package. Either the sequence is further upstream, toward the *leu* region, or RpoS exerts its action indirectly through another control element, such as the *mre* genes (6). As reported previously (3), other PBP changes were also discernible during the shift from

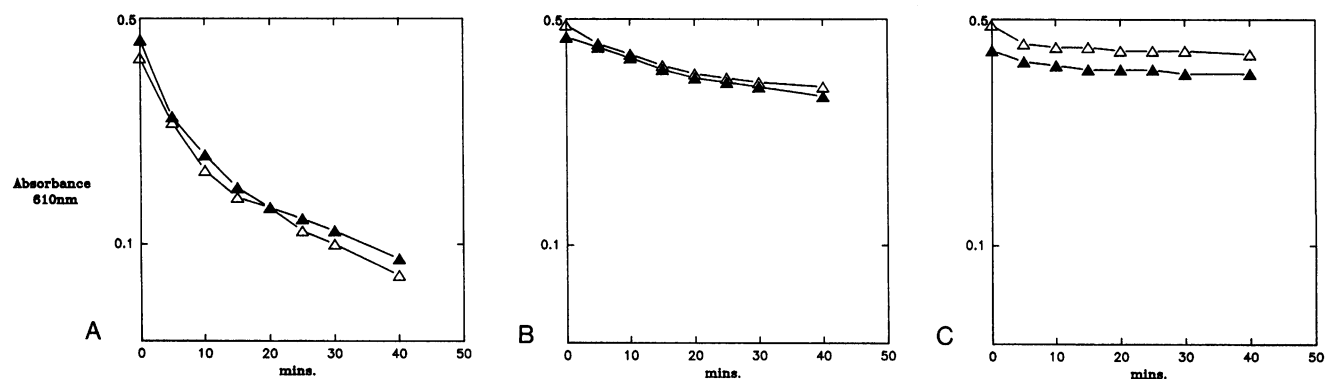


FIG. 2. Autolysis rates of *rpoS*<sup>+</sup> (open triangles) and *rpoS*::kan (solid triangles) *E. coli* cells in the logarithmic, stationary, and overnight phases. The cells were grown in LB at 37°C with shaking. Samples were taken during the logarithmic (A), early stationary (B), and overnight (C) time points and were collected by centrifugation; the pellets were drained of medium and rapidly resuspended in 0.1 M Tris-Cl (pH 8.0)–0.2 mM EDTA at 20°C. After the initial reading at time zero, the cells in buffer were placed at 37°C, and the  $A_{610}$  was read on a Sequoia-Turner model 340 spectrophotometer at the indicated time intervals.

TABLE 4. Peptidoglycan cross-linkage index

Sampling point <sup>a</sup>	Percent cross-linkage <sup>b</sup>	
	<i>rpoS</i> <sup>+</sup>	<i>rpoS</i> ::kan
Logarithmic phase	23.2	22.6
Stationary phase	23.3	23.1
Overnight (24 h)	28.4	28.8

<sup>a</sup> Sample points are identical to those used in Table 1.

<sup>b</sup> Percent cross-linkage was calculated by the formula of Driehuis et al. (8). Values are averages of three determinations.

the logarithmic to the stationary phase; however, these appeared to an equal extent in both the parent and *rpoS*::kan strains.

Bacteria with a disrupted *rpoS* gene appear to be morphologically indistinguishable from wild-type strains during the logarithmic growth phase. Upon entry into the stationary phase, however, the wild-type cells become short coccobacillary forms, while the *rpoS*-disrupted cells are very heterogeneous in cell length (17). Evidence that the PBP 6 change controlled by *BolA* is involved in the short coccobacillary forms has been presented previously (1). Inasmuch as PBP 3 has been associated with septum formation during the process of cell division (29), it is interesting in the present study to find that RpoS appears to downregulate this protein as the cells enter the stationary phase and cease cell division. Prolonged starvation for either a required amino acid or carbon source appeared to have a very similar effect.

The term phenotypic tolerance has been applied to situations in which nongrowing bacteria become refractory to the killing and lytic effects of  $\beta$ -lactam antibiotics (11). With the growing appreciation of the role of RpoS in both the stationary phase and under starvation conditions and the results with the PBPs presented above, it seemed appropriate to test whether RpoS might play a role in phenotypic tolerance. The clear and unambiguous conclusion was that it does not have an influence. Ampicillin-induced cell killing and the development of tolerance upon either entry into the stationary phase or nutrient starvation were totally unaffected by the status of RpoS. This is in contrast to the findings with a stringent control, in which the *relA* gene has been demonstrated to markedly influence cell viability and autolysis (13). Imipenem, which is reported to lyse and kill *E. coli* deprived of an essential amino acid (33), was found to decrease viability by 90% over 3 h in lysine-starved cells. The extent of killing in the *rpoS*::kan strain appeared to be virtually identical to that in the isogenic parent. This indicates that *rpoS* has no direct role in this process.

The cell wall parameters that were measured, autolysis rates and peptidoglycan cross-linkage, were also seemingly unaffected by the *rpoS* disruption. This would indicate that the observed PBP changes are not directly responsible for the development of autolysis resistance and increased cross-linkage in the stationary phase. It is possible, however, that the more detailed examination of the peptidoglycan composition via high-pressure liquid chromatographic analysis would reveal subtle differences not observed by thin-layer chromatography (4, 9). Nonetheless, the physiological mechanisms involved in the peptidoglycan autolysis susceptibility and overall cross-linkage changes in the stationary phase measured in the present study apparently are RpoS independent. Either additional control elements are involved in the cell wall changes that occur upon entry into stationary phase or the changes are passive processes resulting from the decrease in cell growth rate.

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