Induction and Control of the Autolytic System of *Escherichia coli*

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Various methods of inducing autolysis of *Escherichia coli* cells were investigated, some being described here for the first time. For the autolysis of growing cells only induction methods interfering with the biosynthesis of peptidoglycan were taken into consideration, whereas with harvested cells autolysis was induced by rapid osmotic or EDTA shock treatments. The highest rates of autolysis were observed after induction by moenomycin, EDTA, or cephaloridine. The different autolyses examined shared certain common properties. In particular, regardless of the induction method used, more or less extensive peptidoglycan degradation was observed, and 10^{-2} M Mg^{2+} efficiently inhibited the autolytic process. However, for other properties a distinction was made between methods used for growing cells and those used for harvested cells. Autolysis of growing cells required RNA, protein, and fatty acid synthesis. No such requirements were observed with shock-induced autolysis performed with harvested cells. Thus, the effects of Mg^{2+} , rifampicin, chloramphenicol, and cerulenin clearly suggest that distinct factors are involved in the control of the autolytic system of *E. coli*. Uncoupling agents such as sodium azide, 2,4-dinitrophenol, and carbonyl-cyanide-*m*-chlorophenyl hydrazone used at their usual inhibiting concentration had no effect on the cephaloridine or shock-induced autolysis.

The conditions determining the stability of *Escherichia coli* cells, either in growth medium or after harvest, have not so far been extensively investigated. Generally, cells of wild-type strains of *E. coli* do not lyse spontaneously at a high rate, and some kind of induction is necessary to bring about rapid autolysis. Although different methods of promoting autolysis of *E. coli* have been reported (19, 21, 27, 30, 34), no comparative study has yet been undertaken, and the mechanisms involved are poorly understood. It is generally assumed that one of the main steps in the autolytic process is the degradation of peptidoglycan by specific endogenous hydrolases (32, 37, 41, 48). A closer investigation of the induction and control of the autolytic system of *E. coli* could perhaps help to determine which peptidoglycan hydrolases play a major role in this process, what their physiological functions are during the formation of peptidoglycan, and how their action is controlled.

The aim of the present report was to compare the properties of autolysis induced in *E. coli* cells by various methods, some being described here for the first time. A distinction was made between methods used for cells in growth medium and those used for harvested cells. Furthermore, the influence of different cellular functions on the induction of autolysis was examined

in an attempt to determine the mechanisms involved in the control of the autolytic system of *E. coli*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Most experiments were carried out with *E. coli* K-12 HfrH (21). *E. coli* W7 (Lys⁻ DAP⁻) and *E. coli* EH3247 (GlcN⁻) were kindly provided by C. Lazdunski (C.B.B.M., Centre National de la Recherche Scientifique, Marseille, France) and M. Sarvas (University of Helsinki, Helsinki, Finland) respectively. *E. coli* B and *E. coli* K235 were ATCC strains. *E. coli* H1119 is a temperature-sensitive lysis mutant impaired in the synthesis of cytoplasmic peptidoglycan precursors and was kindly provided by B. Lugtenberg (Utrecht, The Netherlands). Strains K-12, W7, B, and K235 were grown in rich or minimum medium as described previously (21). Strain EH3247 was grown in 1% yeast extract with *N*-acetylglucosamine (100 µg/ml) (33). Strain H1119 was grown at 30°C as described by Lugtenberg et al. (22). All cultures were carried out with 40 ml of medium in 200-ml flasks. Growth was followed by measuring the optical density at 600 nm in a Gilford spectrophotometer (model 240).

Antibiotics and reagents. Penicillin G was purchased from Calbiochem (Hoescht, Paris), carbonyl cyanide-*m*-chlorophenyl hydrazone and *p*-hydroxymercuribenzoate, sodium salt, was from Serva (Heidelberg, Federal Republic of Germany), 2,4-dinitrophenol was from Sigma (Saint Louis, Mo.), and D-cycloserine was from

Roche (Paris). 11,837 RP was a gift from Rhône-Poulenc (Vitry, France), cephaloridine was a gift from Eli Lilly (St-Cloud, France), and moenomycin was a gift from Hoescht AG (Frankfurt, Federal Republic of Germany).

Autolysis procedures. The induction of autolysis of harvested cells by shock treatments with EDTA or sodium phosphate was carried out by the method described previously for the osmotic shocks with water and sodium acetate (21). Early exponential-phase cells (ca. 2×10^8 cells per ml) were harvested by centrifugation at room temperature (25°C) for 5 min at $12,000 \times g$. After the supernatant fluid was discarded, the inside of the centrifuge tube was wiped carefully with cotton tips to remove as much growth medium as possible. The unwashed pellet was immediately suspended by vigorous agitation at room temperature in 20 ml of 10^{-3} M EDTA (pH 6.5) or 5×10^{-2} M sodium phosphate (pH 7). After absorbance measurements at zero time, the suspension was transferred to a 200-ml flask and placed at the appropriate temperature in a water bath without shaking.

Inductions in the growth medium were performed with early exponential-phase cells (ca. 10^8 cells per ml) to which the concentrated solution (100 μ l) of antibiotic was added, and cultures were maintained with shaking throughout the autolytic process. Autolysis of strains W7 and EH3247 was induced by harvesting quickly at room temperature early exponential-phase cells and resuspending them in fresh medium deprived of either *N*-acetylglucosamine or α '-meso-diaminopimelic acid (DAP) (28, 33). Autolysis of strain H1119 was triggered by shifting early exponential-phase cultures from 30 to 42°C (22). In all cases cell lysis was followed by measuring the decrease of turbidity at 600 nm with time. The first-order rate constant of autolysis was calculated as previously described (21).

Treatment with uncoupling agents. To early exponential-phase cells of *E. coli* K-12 grown in rich medium various concentrations of uncoupling agents such as 2,4-dinitrophenol (0.5×10^{-3} to 2×10^{-3} M), sodium azide (1×10^{-3} to 40×10^{-3} M), or carbonyl cyanide *m*-chlorophenyl hydrazone (1×10^{-6} to 100×10^{-6} M) were added. Induction of autolysis by cephaloridine (5 μ g/ml) was performed 5 min after the addition of the drug, and induction of autolysis by osmotic shock (water-sodium acetate or EDTA) was performed 10, 20, or 30 min after the addition of the drug.

Measurement of peptidoglycan hydrolysis. Cells of *E. coli* W7 were grown in presence of 10^{-4} M [3 H]DAP (specific activity, 60 mCi/mmol; CEA, Saclay, France). Cells were subjected to autolysis in growth medium or after harvesting. At different time intervals three 0.5-ml samples were suspended in 5% trichloroacetic acid containing 10^{-4} M DAP and left for 1 h at 0°C. The suspensions were then filtered on membrane filters (0.45- μ m pore size; Millipore Corp., Bedford, Mass.), and radioactivity was counted (21).

RESULTS

Induction of autolysis. When the autolysis of *E. coli* cells was induced in the growth medium, only methods involving the specific blocking of steps of peptidoglycan synthesis were considered. It is a well-established fact that interfer-

ence with the synthesis of this macromolecule in *E. coli* leads to cell lysis (46). The various methods used in the present work were as follows: (i) treatments with antibiotics such as D-cycloserine (5), beta-lactams (46), and moenomycin; (ii) deprivation of DAP or glucosamine in strains auxotrophic for this amino acid (28, 36) or hexosamine (33); and (iii) transfer of strains with a thermosensitive mutation in a step of peptidoglycan synthesis to the nonpermissive temperature (22).

Beta-lactam antibiotics can efficiently induce lysis of growing cells of *E. coli* (19, 46). Their cellular targets are minor cytoplasmic membrane proteins designated as penicillin-binding proteins (40). Apparently, in *E. coli* the binding of beta-lactams to PBP-1a and PBP-1b most probably brings about autolysis (6, 19, 40, 42, 44). Among these antibiotics, cephaloridine preferentially binds to these PBPs and causes rapid lysis at the lowest concentration. PBP-1a and PBP-1b appear to be multifunctional enzymes capable of catalyzing both the transglycosylation and transpeptidation reactions leading to polymerized peptidoglycan material (16, 26, 43, 45). Whereas the transpeptidation reactions are the preferential targets of certain beta-lactams, antibiotics belonging to the moenomycin family (39, 49) are specific inhibitors of the transglycosylation reactions (16, 43, 47). Antibiotics such as moenomycin or 11,837 RP on *E. coli* K-12 were extremely efficient in promoting high-rate autolyses (Fig. 1 and 2).

The autolysis of unwashed harvested exponential-phase cells of *E. coli* could be efficiently brought about by different osmotic shock treatments. The induction by associating rapid shocks with water and sodium acetate has been previously described in detail (21). We observed that autolysis is also readily promoted by a shock treatment with EDTA (10^{-3} M) or sodium phosphate ($5 \cdot 10^{-2}$ M) (Fig. 1b). A shock with 5×10^{-2} M NaCl alone had no lytic effect. Other EDTA treatments leading to partial cell lysis have been reported (38). It should be stressed that autolysis is efficiently brought about by the shock treatments only when cells are rapidly harvested at room temperature, the culture medium is thoroughly eliminated, and the unwashed cell pellet is very quickly and vigorously suspended in the shock medium. The autolysis of wild-type strains such as *E. coli* B and K235 was also efficiently promoted by these techniques. A certain variability of the rate and extent of phosphate-induced autolysis was observed with different *E. coli* K-12 strains.

It should be noted that, either with growing cells or harvested cells, the rate and extent of autolysis depended greatly on the induction method used. The highest rates were observed

FIG. 1. Kinetics of autolysis of *E. coli*. (A) Effect of moenomycin (10 µg/ml) (■) or cephaloridine (5 µg/ml) (○) on early exponential-phase cells (10^8 cells per ml) of *E. coli* K-12 HfrH grown at 37°C in rich medium and (▽) lysis of *E. coli* W7 (DAP⁻ Lys⁺) in rich medium after DAP deprivation. (B) Early exponential-phase cells (2×10^8 cells per ml) grown at 37°C in rich medium were harvested, and autolyses were induced by shock treatment as described in the text. Shock treatments with (▽) 0.05 M sodium chloride (pH 7); (■) distilled water and 0.5 M sodium acetate (pH 6.5); (○) 10^{-3} M EDTA (pH 6.5); (▽) 0.05 M sodium phosphate (pH 7).

(Fig. 1 and 2) with moenomycin ($k = 220 \times 10^{-3}/\text{min}$ with 10 µg/ml), the EDTA shock ($k = 147 \times 10^{-3}/\text{min}$), and cephaloridine ($k = 130 \times 10^{-3}/\text{min}$ with 5 µg/ml).

Effect of growth conditions and temperature on autolysis. The initial rate of autolysis of *E. coli* cells is dependent on growth conditions, apparently whatever the induction method considered (Table 1). The rate of autolysis is greatest for fast-growing cells. Stationary-phase cells autolyse slowly or not at all. Similar results were found with the osmotic shock-induced and beta-lactam-induced lyses of *E. coli* cells (2, 11, 21, 25). It was previously shown that osmotic shock-induced autolysis is dependent not only on growth conditions but also on the temperature at which cells are incubated during autolysis (21). This same dependency was observed for lyses promoted by various methods (Table 1).

Effect of Mg^{2+} . Regardless of the method used for inducing autolysis, the addition of 10^{-2} M MgSO_4 had a complete inhibitory effect on cell lysis (Fig. 2). Owing to the absence of a lag period between induction and autolysis in the case of the osmotic shocks, Mg^{2+} was added in the course of the autolytic process. With the other induction methods, Mg^{2+} was added just 5 min before the onset of expected lysis and also in the course of autolysis. It has been shown previously (21) that the addition of Mg^{2+} to autolyzing cells after induction by osmotic shock (water-sodium acetate) also immediately arrested loss of viability. Such comparisons have not yet been made with the other induction methods. Important increases of optical density with time were observed in a few instances (Fig.

2e, f, and h) after the addition of Mg^{2+} . It is noteworthy that in the case of *E. coli* EH3247 a doubling of the optical density before leveling off has already been observed after removal of glucosamine under stabilizing conditions and was associated with an increase in cell size (33). Presently, we have not further studied the increases observed with the penicillin- or cephaloridine-induced autolysis.

Influence of different cellular functions on autolysis of *E. coli*. When considering the effects of the inhibition of RNA, protein, or fatty acid synthesis on autolysis, important differences were observed between the induction of the autolytic system of growing cells and that of harvested cells. Pretreatment with rifampicin, chloramphenicol, or cerulenin (4) had little effect on the course of the shock-induced autolyses carried out only with harvested cells (Fig. 3). However, these pretreatments greatly reduced the rate and extent of the cephaloridine and moenomycin-induced autolyses of growing cells (Fig. 4). The effect was more strongly marked with cephaloridine-induced lysis than with moenomycin-induced lysis.

The role of uncoupling agents (12) such as 2,4-dinitrophenol, sodium azide, and carbonyl cyanide-*m*-chlorophenyl hydrazone on the induction of autolysis of *E. coli* K-12 cells was investigated. At their usual inhibiting concentrations (12) these drugs had no effect on the rate and extent of autolysis induced by either cephaloridine or osmotic shocks (water-sodium acetate or EDTA). These results with low concentrations of 2,4-dinitrophenol and sodium azide are in agreement with those of Mohan et al. (30).

At higher concentrations, sodium azide (2×10^{-2} M) blocked cephaloridine-induced autolysis, but not osmotic shock-induced autolysis, whereas 2,4-dinitrophenol had little effect in

both cases. It was also observed that 10^{-4} M carbonyl cyanide-*m*-chlorophenyl hydrazone promoted the autolysis of harvested *E. coli* cells and to a lesser extent that of cells growing in

FIG. 2. Effect of Mg^{2+} ions on autolysis of *E. coli* after induction by the following: (a) 10^{-3} M EDTA (pH 6.5); (b) 0.05 M sodium phosphate (pH 7); (c) moenomycin (5 μ g/ml) or 11,837 RP (5 μ g/ml); (d) 4×10^{-4} M D-cycloserine, (e) cephaloridine (5 μ g/ml); (f) penicillin G (50 μ g/ml); (g) *E. coli* W7; (h) *E. coli* EH3247; (i) *E. coli* H 1119. All inductions of autolyses were at $t = 0$; 10^{-2} M $MgSO_4$ was added as indicated by the arrow ($\downarrow$). Symbols: untreated cells ($\square$); Mg^{2+} -treated cells ($\bullet$).

TABLE 1. Effect of growth conditions and temperature of lysis on the initial rate constant k of autolysis of *E. coli* K-12 HfrH^a

Growth phase	Medium	Temp of autolysis (°C)	k after treatment with:			
			EDTA	Cephaloridine	Moenomycin	D-Cycloserine
Exponential	Rich	37	0.147	0.130	0.220	
		25	0.077	0.065	0.056	
Stationary	Rich	37	0.020	0	0	
Exponential	Minimum	37	0.007	0	0	0.037
Stationary	Minimum	37	0	0	0	0

^a Autolyses were induced either with harvested cells by EDTA shock or with cells in growth medium by cephaloridine (5 μ g/ml), moenomycin (10 μ g/ml), or D-cycloserine (4×10^{-4} M). For lyses in growth medium, the influence of temperature was observed after transferring flasks to 25°C, 5 min before the onset of autolysis. Generation times in rich and minimum media were 35 and 60 min, respectively.

medium. At the concentration used, carbonyl cyanide-*m*-chlorophenol hydrazone probably does not act only as a protonophore, but according to Kaback et al. (17) most likely acts as a sulfhydryl reagent. Specific sulfhydryl reagents such as *p*-hydroxymercuribenzoate have been shown to induce autolysis of *E. coli* B/r (34). We found that 10^{-5} M *p*-hydroxymercuribenzoate had a similar effect on *E. coli* K-12 harvested cells. Preliminary experiments with potassium cyanide (10^{-2} M) showed no effect on osmotic shock-induced lysis, but seemed to block cephaloridine-induced lysis.

Correlation between autolysis and peptidoglycan degradation. A close correlation between peptidoglycan degradation and autolysis induced by shock treatment with water and sodium acetate (21) has already been established. It has also been observed that some extent of

peptidoglycan degradation accompanied penicillin- and cephaloridine-induced autolyses (9, 19). By using [³H]DAP-labeled *E. coli* W7 as previously described (21), a similar relationship was now observed for some of the other types of autolyses considered here (Table 2). It is noteworthy that the rates of peptidoglycan degradation were highest for the shock induced-autolyses, and that in these cases they more or less paralleled the decrease in optical density.

DISCUSSION

Although very different methods of promoting autolysis of *E. coli* were considered in the present paper, the resulting lytic processes shared certain properties in common (rate dependency on growth conditions, temperature, and Mg^{2+} and degradation of peptidoglycan). In particular, peptidoglycan degradation seemed to be associ-

FIG. 3. Effect of rifampicin, chloramphenicol, and cerulenin on EDTA shock-induced autolysis of *E. coli* K-12 HfrH grown in rich medium at 37°C. Early exponential-phase cells (2×10^8 cells per ml) were treated during growth with either (A) rifampicin (10 μ g/ml) for 20 min, (B) chloramphenicol (100 μ g/ml) for 20 min, or (C) cerulenin (100 μ g/ml) for 30 min before harvesting. The harvested cells were submitted to the EDTA shock. Symbols: untreated cells (■); treated cells (□). Longer treatment (1 h) with rifampicin or chloramphenicol gave results identical to those with the 20-min treatments. Furthermore, it should be noted that rifampicin arrested growth only after a 45-min treatment.

FIG. 4. Effect of rifampicin, chloramphenicol, and cerulenin on moenomycin- and cephaloridine-induced autolyses of *E. coli* K-12 HfrH cells growing in rich medium at 37°C. Early exponential-phase cells (10^8 cells per ml) were untreated ($\square$) or treated with either rifampicin (10 $\mu\text{g/ml}$) for 20 min ($\circ$), chloramphenicol (100 $\mu\text{g/ml}$) for 20 min ($\bullet$), or cerulenin (100 $\mu\text{g/ml}$) for 30 min ($\blacktriangledown$) before the addition of the inducing agent, (A) moenomycin (10 $\mu\text{g/ml}$) or (B) cephaloridine (5 $\mu\text{g/ml}$). It was verified that protein synthesis continued unabated for 40 min after the addition of cerulenin.

ated with the different autolyses examined, thus implying the involvement of peptidoglycan hydrolases in the autolytic process. However, the extent of peptidoglycan degradation was clearly less when induction of autolysis was carried out with growing cells (cephaloridine, moenomycin, or DAP deprivation) than with the shock-induced lyses of harvested cells. Moreover, the decreases in optical density were similar or, in fact, greater in the former cases. This is presumably due to differences in the specificity, activity, and location of the peptidoglycan hydrolases involved in the degradation process. It should also be stressed that owing to the thinness of the peptidoglycan of *E. coli* (3), even a limited degradation should lead to rapid cell lysis.

Among the numerous peptidoglycan hydrolases of *E. coli* (32), it is not yet clear which ones are directly involved in the early stages of the autolytic process. Recently, Kitano et al. (20) have isolated beta-lactam-tolerant mutants of *E. coli* defective in their autolytic activity and presenting reduced peptidoglycan glycosidase and endopeptidase activities. Perhaps these two activities play a major role in autolysis. The control of such activities and the induction of

autolysis are undoubtedly two closely related matters. As far as *E. coli* is concerned, the results presented here clearly showed that a

TABLE 2. Extent of peptidoglycan degradation for various autolyses

Autolysis	% of optical density decrease at 600 nm ^a	% of peptidoglycan degradation ^{a,b}
Water-sodium acetate, 0.5 M, pH 6.5	31	35
EDTA, 10^{-3} M, pH 6.5	69	66
Sodium phosphate, 5×10^{-2} M	44	45
Moenomycin	73	28
Cephaloridine	82	23
<i>E. coli</i> W7 (DAP ⁻ Lys ⁻)	58	26

^a Measurements after 1 h of autolysis.

^b Percentage of solubilized peptidoglycan after 1 h of autolysis as estimated by the amount of [^3H]DAP remaining in the trichloroacetic acid-insoluble material.

distinction must be made between fast-growing cells in medium and harvested cells.

Weidel and Pelzer (48) have proposed that during growth of *E. coli* cells the continuous cleavage of peptidoglycan bonds is required for the enlargement of the envelope and that a well-adjusted balance exists between peptidoglycan hydrolases and enzymes catalyzing the insertion of newly polymerized material. Therefore, any abrupt arrest of peptidoglycan synthesis during fast growth will lead to an unbalanced state where hydrolases continue unabated to cleave peptidoglycan linkages. The result is cell lysis. Under such circumstances autolysis is not due to any specific induction of hydrolases, but rather to a delay in the switching on of appropriate control mechanisms. Thus, according to this scheme the interference with any step in peptidoglycan synthesis causes the depletion of the precursors of the subsequent steps. Owing to the rapid turnover of the peptidoglycan precursor pools (29), the indirect and ultimate effect of such an inhibition is the rapid arrest of the polymerization reactions and the efficient induction of autolysis.

The harvesting of cells, with their segregation from the medium and the stop of aeration, can be considered as a down-shift. Under such conditions RNA and protein syntheses are very rapidly stopped (24). Harvested cells are thus somewhat like growing cells treated with rifampicin or chloramphenicol. Apparently, in both cases the arrest of protein synthesis puts some kind of control on the autolytic system. It has been stressed that a specific mechanism must be operating to slow down or shut off autolytic activity during general starvation (48). This is confirmed by the fact that rifampicin or chloramphenicol had little effect on the shock-induced autolyses carried out with harvested cells. The shock treatment of harvested cells can thus be considered as a true induction of the autolytic activity.

The existence of mechanisms regulating peptidoglycan hydrolases of *E. coli* has been recognized and expressed in terms of a barrier by Hartmann et al. (13). The recent study of mutant $\chi 2452$ of *E. coli* (18) is in favor of a specific control of the autolytic system of *E. coli*. The data presented here on the effects of Mg^{2+} , rifampicin, chloramphenicol, and cerulenin clearly suggest that perhaps several distinct factors are involved in this control.

Whatever the induction method considered, Mg^{2+} efficiently inhibited autolysis. McQuillen has shown that Mg^{2+} has a dramatic effect in preventing penicillin-induced lysis (27). It was speculated (21) that the removal of Mg^{2+} by shock treatments is an essential step in the induction of autolysis of harvested cells. The

possible role of this cation in the control of autolysis has already been discussed in detail (21). One possibility is that Mg^{2+} has a direct effect on the peptidoglycan hydrolases involved in the autolytic process. Another possibility is that the control of these hydrolases is dependent on the state of the envelope as a whole and that the removal of divalent cations from the cell induces some disorganization of the envelope, which in turn triggers the uncontrolled action of peptidoglycan hydrolases by a yet-unknown mechanism. Different observations partially substantiate this latter point of view (21). It is noteworthy that in the case of osmotic shock (water-sodium acetate)-induced lysis Mg^{2+} arrested both autolysis and peptidoglycan degradation. Such comparisons have not yet been made with other types of autolysis.

The requirement of protein synthesis for the autolysis of *E. coli* growing cells has been reported in many instances (9, 19, 23, 27, 28, 31, 36). To explain this dependency, Goodell and Tomasz (9) have recently proposed that inhibition of protein synthesis causes a rapid modification of peptidoglycan structure in such a way that attachment of peptidoglycan hydrolases is inhibited. However, this does not explain why shock treatments efficiently induce the autolytic system in the absence of protein synthesis. These experiments also show that there is no rapid inactivation of the autolytic activities of *E. coli*, at least under the conditions in which the cells are harvested. A requirement for fatty acids synthesis was found with growing cells of *E. coli*. As far as we are aware, it is the first time that such a control has been observed.

In the present work we have focused our attention on certain induction methods. However, it should be stressed that autolysis of *E. coli* cells can be promoted under many other conditions, e.g., use of chaotropic reagents (15), phage infection, etc. Furthermore, greatly increased susceptibility to cell lysis has been encountered in mutants of *E. coli* deficient in the organization of the cell envelope (14, 50) or in other cellular functions (1). In some cases, Mg^{2+} , rifampicin, and chloramphenicol have been shown to have effects similar to those described here (10, 14, 15, 50).

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