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Evidence for a Role of *N*-Acetylmuramyl-L-Alanine Amidase in Septum Separation in *Escherichia coli*

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Septum formation and septum separation have been studied in a chain-forming mutant of *Escherichia coli* K-12 bearing the *envA* mutation and its parental strain. In comparison to the wild type, the mutant showed a sixfold reduction in the specific activity of the enzyme, *N*-acetylmuramyl-L-alanine amidase (EC 3.5.1.28), part of which was associated to the outer membrane. Genetic as well as physiological suppression of chain formation resulted in an increase in amidase activity. The addition of *N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid to growing wild-type cells and to cells bearing the *envA* mutation caused an inhibition of cell separation and an increased frequency of visible septa. The kinetics of septum formation and separation was followed in chains by the use of ampicillin and nalidixic acid. The latter drug inhibited initiation of new septa but allowed preformed ones to go to cell separation at a rate corresponding to that of steady-state growing cells. Ampicillin treatment, on the other hand, resulted in a more rapid decrease in the frequency of septa. The disparate effects of ampicillin and nalidixic acid were not explained by a difference in amidase activity but could be due to an inhibitory effect of ampicillin on a septal peptidoglycan fusing activity.

With common fixation techniques septa are rarely visualized in wild-type *Escherichia coli* K-12. Burdett and Murray have suggested that the constrictions observed in these cells are artifacts due to residual autolysis during fixation (3). Mutants of *E. coli* K-12 in which septa are stable (15, 19) have been isolated. One such mutant bearing the *envA* mutation forms chains of cells during growth in rich medium, where each cell unit is delimited by a partial or fully developed septum (15). The complete septum consists of cytoplasmic membrane, periplasm, and two layers of fused peptidoglycan.

In cells carrying the *envA* mutation septum formation involves an ingrowth of the cytoplasmic membrane and peptidoglycan perpendicular to the longitudinal axis. Cell division is then accomplished by the invagination of outer membrane separating the fused peptidoglycan layers (15). The association of the septal peptidoglycan appears covalent in nature. Therefore, in cells bearing the *envA* mutation a defect in septal peptidoglycan-splitting activity may be responsible for chain formation.

In *Bacillus subtilis* evidence has been collected for a role of *N*-acetylmuramyl-L-alanine amidase in septum separation (4, 5). In *Streptococcus pneumoniae* the main autolytic enzyme *N*-acetylmuramyl-L-alanine amidase can be inhibited by replacing choline for ethanolamine in the growth medium (9). Under these conditions the bacteria grow into long chains with fully compartmentalized cells (21).

Also *E. coli* contains an *N*-acetylmuramyl-L-alanine amidase (23) that was recently purified to near homogeneity (22). The enzyme was suggested to be periplasmic or associated to the outer membrane. However, no data that could indicate the biological function of the enzyme were given. In this report we provide evidence for the involvement of *N*-acetylmuramyl-L-alanine amidase in the cell separation process of *E. coli* K-12, using the chain-forming mutant D22 (*envA*), which exhibits a reduced amidase activity.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *E. coli* K-12 strains D21 (*envA*$^+$), D22 (*envA*), and D22S1 (*envA sup-200*) have been described previously (13). The complete medium was the LB medium of Bertani (1) supplemented with medium E of Vogel and Bonner (24), thiamine, and 0.2% glucose. The Casamino Acids medium contained medium E plus thiamine, 0.2% casein hydrolysate supplemented with L-tryptophan (50 μg/ml), and 0.2% glucose. The minimal medium contained medium E plus B_1, 0.2% glucose, and 50 μg each of L-proline, L-tryptophan, and L-histidine per ml. The growth was

carried out at 37°C and recorded as the absorbance reading at 450 nm in a Zeiss spectrophotometer.

Materials. D-Ampicillin was provided by AB Astra, Södertälje, Sweden. Nalidixic acid was purchased from Winthrop Ltd., Surbiton on Thames, England. Cellulose thin-layer chromatography plates (Kodak) were from Kebo AB, Stockholm, Sweden. *meso*-[^{3}H]diaminopimelic acid (DAP) (300 mCi/mmol) and L-[^{14}C]alanine (135 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.

Purification of *N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP, *N*-acetylmuramyl-L-alanyl-D-glutamic acid, and *N*-acetylmuramyl-L-alanine. *N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP was prepared from *Bacillus cereus* as recently described (H. Wolf-Watz, Ph.D. thesis, University of Umeå, Umeå, Sweden, 1976). Normally a 2-liter culture was grown at 37°C in rich medium supplemented with *meso*-DAP. At a density of 100 Klett units the cells were harvested, washed once in saline, and suspended in 200 ml of a 10× concentrated cell wall medium as described by Park and Chatterjee (18) and supplemented with 1 mM [^{3}H]DAP (5 mCi/mmol; 3,600 cpm/nmol). However, vancomycin was replaced by D-cycloserine (100 μg/ml) (10). After incubation at 37°C for 90 min the cells were recovered by centrifugation and suspended in 20 ml of ice-cold 10% trichloroacetic acid. After extraction for 70 min on ice the precipitated material was removed by centrifugation, and the supernatant fluid containing low-molecular-weight components was extracted three times with ether to remove the trichloroacetic acid. A 50-ml volume of ethanol containing potassium acetate (0.5%, wt/vol) was added.

After centrifugation (40,000 × *g*, 15 min) the supernatant fraction was concentrated to dryness in a rotary evaporator. This material was suspended in 2 ml of 0.05 M HCl and hydrolyzed at 100°C for 5 min (23). The entire hydrolysate was applied to a Sephadex G25 column (2 by 80 cm) equilibrated with 0.01 M acetic acid. After elution (3.8-ml fraction) two *meso*-[^{3}H]DAP-containing peaks were identified (fractions 50 to 68 and fractions 72 to 100, respectively). In addition, both peaks contained hexosamine.

After lyophilization of fractions 72 to 100 the material was dissolved in 2 ml of 0.01 M acetic acid and chromatographed using the same conditions as described above. The *meso*-[^{3}H]DAP was eluted between fractions 70 to 90, whereas detectable amounts of hexosamine were eluted between fractions 60 and 80. After lyophilization of fractions 72 to 80, the material was solved in a small volume of distilled water to a final concentration of 4 mM *meso*-DAP. The material obtained was used in all enzyme assays as a substrate for *N*-acetylmuramyl-L-alanine amidase.

N-acetylmuramyl-L-alanyl-D-glutamic acid and *N*-acetylmuramyl-L-alanine analogous to *N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP were prepared as described above by omitting from the cell wall medium *meso*-DAP and D-glutamic acid, respectively. To trace *N*-acetylmuramyl-L-alanyl-D-glutamic acid and *N*-acetylmuramyl-L-alanine the molecules were labeled with L-[^{14}C]alanine (0.25 μCi/μmol; 238 cpm/nmol).

Amidase assay. Crude extracts were used in the standard assay for *N*-acetylmuramyl-L-alanine amidase. A 100-ml culture containing about 3 × 10^8 cells/ml was harvested by centrifugation. The cells were suspended in 5 ml of 0.1 M sodium phosphate buffer (pH 8.0), and the cells were broken by sonic oscillation. Intact cells were removed by centrifugation (3,000 × *g* for 20 min), and the supernatant fluid was used as crude enzyme extract. The assay mixture contained 1.5 μmol of sodium phosphate (pH 8.0) and 20 nmol of *N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-[^{3}H]DAP (3,600 cpm/mmol) in a total volume of 20 μl. The mixture was incubated at 37°C for 60 min, and the reaction was terminated by cooling to 0°C. The products of the reaction were separated by electrophoresis on cellulose thin-layer chromatography plates (2 by 20 cm) in 0.1 M formic acid using 25 V/cm at 60 min. The radioactive spots were located and counted in a Nuclear-Chicago Mark II liquid scintillation counter. The specific activity of *N*-acetylmuramyl-L-alanine amidase was given as nanomoles of product formed per hour per milligram of protein. Protein was estimated by the method of Lowry et al. (11) using crystalline bovine serum albumin as standard.

Determination of cell number and septa. Samples of cells were immediately chilled and fixed in 0.05% formaldehyde solution. Cell number was determined with a Petroff-Hausser bacteria counter chamber. A chain as well as a filament was always counted as one cell. The frequency of septa was determined under a Zeiss phase-contrast microscope.

RESULTS

Deficient activity of *N*-acetylmuramyl-L-alanine amidase in the septum separation-defective mutant D22 (*envA*). The specific amidase activity of crude extracts was measured in growing LB cultures of the chain-forming mutant D22 (*envA*), its parental strain D21, and the spontaneous *envA*$^+$ revertant D22S1 (Table 1). A sixfold reduction of the specific activity in D22 (*envA*) as compared to the other strains was noted. Moreover, no measurable activity was found in the culture fluid.

Enzyme extracts from strains D21 (*envA*$^+$) and D22 (*envA*) were mixed in different ratios (Table 2). No striking differences in activity between mixed extracts and calculated values were observed.

Association of the *N*-acetylmuramyl-L-alanine amidase to the outer membrane. Cytoplasmic and outer membrane of strain D21 and D22 (*envA*) was separated according to the method of Osborn et al. (17). The activity of amidase was assayed in the different membrane fractions. In both strains a significant

TABLE 1. *Specific activity of N-acetylmuramyl-L-alanine amidase in envA and envA⁺ strains of E. coli K-12*[a]

Strain	Genotype	*N*-acetylmuramyl-L-alanine amidase (nmol/h per mg of protein)
D21	*envA*⁺	8.5
D22	*envA*	1.5
D22S1	*envA sup-200*	7.9

[a] Crude enzyme extracts were prepared from cells growing exponentially in LB medium.

TABLE 2. *Specific activity of N-acetylmuramyl-L-alanine amidase in mixed extracts of strain D21 (envA⁺) and D22 (envA)*[a]

Protein (μg) in the reaction mixture derived from:		Sp act of *N*-acetylmuramyl-L-alanine amidase (nmol/h per mg of protein)	
D21	D22	Found activity	Expected activity[b]
115	0	7.9	8.5[c]
103	10	7.6	7.9
31	36	4.9	4.7
16	56	2.3	3.0
6	68	1.9	2.1
0	75	1.5	1.5[c]

[a] Crude enzyme extracts were prepared from D21 and D22 cells growing exponentially in LB medium. Extracts were mixed in different ratios and the activity of amidase was measured.

[b] Expected amidase activity in mixed extracts if no activators or inhibitors are present.

[c] Mean values of amidase activity in strains D21 and D22.

amount (50%) of the total amidase activity was localized to the total membrane fraction (Table 3). Of the total activity about 30 to 40% was found in the outer membrane fraction, whereas only 5 to 7% was traced to the cytoplasmic membrane fraction (Table 3). The specific amidase activity of outer membrane prepared from strain D22 (*envA*) was significantly lower than that of the parental strain D21.

Correlation between frequency of septa and the activity of *N*-acetylmuramyl-L-alanine amidase. In the wild-type strain D21 the specific activity of *N*-acetylmuramyl-L-alanine amidase was not significantly altered within the growth rate 2.6 and 1.2 doublings/h (Table 4). In contrast, in strain D22 (*envA*) a decrease in growth rate was accompanied by a considerable increase in amidase activity (Table 4). Therefore, in minimal glucose medium the specific amidase activity in strain D22 (*envA*) approached that of the wild type. The mutant D22 (*envA*) forms chains during growth in rich LB medium, where the mean chain exhibits three complete or partial septa. The frequency of septa per cell, however, decreased markedly when the growth rate was lowered (Table 4).

Phenethyl alcohol (PEA) at low concentrations partially suppresses the EnvA phenotype, i.e., chain formation and antibiotic hypersensitivity (14). The low concentrations used (0.025% to 0.05%, vol/vol) have no significant effect on the growth rate. When strain D22 (*envA*) was grown at steady-state conditions in rich LB medium supplemented with 0.05%, vol/vol, PEA, the frequency of septa was 0.59 per chain as compared to 2.87 for untreated mutant cells (Table 5). In contrast in the wild-type strain, the drug increased the frequency of septa per

TABLE 3. *Localization of N-acetylmuramyl-L-alanine amidase in different membrane fractions of strains D21 (envA⁺) and D22 (envA)*[a]

Strain	Membrane fraction	*N*-acetylmuramyl-L-alanine amidase	
		Sp act (nmol/h per mg of protein)	% of total activity
D21 (*envA*⁺)	Total membrane	7.7	52
	Cytoplasmic membrane	2.1	5
	Outer membrane	10.2	35
D22 (*envA*)	Total membrane	1.6	56
	Cytoplasmic membrane	0.7	7
	Outer membrane	4.1	30

[a] Membrane fractions were prepared from cells grown in LB medium according to the method of Osborn et al. (17). Enzyme assays were carried out as described in Materials and Methods.

TABLE 4. *Specific activity of N-acetylmuramyl-L-alanine amidase and frequency of septa in envA⁺ and envA cells grown at different rates*[a]

Strain	Growth rate (doublings/h)	Sp act of amidase (nmol/h per mg of protein)	Frequency of septa per cell
D21 (*envA*⁺)	2.6[b]	8.5	0.25
	1.9[c]	10.4	0.17
	1.2[d]	9.7	0.15
D22 (*envA*)	1.9[b]	1.5	2.87
	1.7[c]	4.6	1.77
	1.1[d]	7.0	0.68

[a] Crude enzyme extracts were prepared from cells growing in the exponential phase.

[b] LB medium was used.

[c] Casamino Acids were used.

[d] Minimal glucose medium was used.

TABLE 5. *Effect of PEA on amidase activity and frequency of septa in strains D21 (envA+) and D22 (envA)*[a]

Strain	Growth rate (doublings/h)	Sp act of amidase (nmol/h per mg of protein)	Frequency of septa per cell
D21	2.6	8.5	0.25
D21 + PEA	2.5	3.3	0.42
D22	1.9	1.5	2.87
D22 + PEA	1.8	3.6	0.59

[a] Cells were grown in steady state in LB medium or in LB medium supplemented with PEA (0.05%, vol/vol). Enzyme assays were performed as described in Materials and Methods.

cell unit from 0.25 to 0.42. In strain D22 the suppression of chain formation by PEA was accompanied by an increase in amidase activity from 1.5 to 3.6. In the wild-type D21 the drug caused a decrease in amidase activity from 8.5 to 3.3.

Effect of *N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP on septum separation in strain D21 (*envA*+) and D22 (*envA*). When the *N*-acetylmuramyl-tripeptide was added to wild-type D21 cells, an immediate inhibition of cell division was observed. Increase in cell mass continued at an unchanged rate for another 25 min, after which a decrease in growth rate was recorded (Fig. 1). The observed inhibition of cell separation was associated with a doubling in the frequency of visible septa per cell. The *N*-acetylmuramyl-tripeptide was also added to growing *envA* cells (Fig. 2). An immediate decrease in growth rate was noticed. Moreover, no cell separation occurred during the entire experiment. Most significant was the observed increase in the frequency of septa per chain (from 2.5 to 5.8). Neither the commercial compounds (*N*-acetylmuramic acid, L-alanine, D-glutamic acid, and *meso*-DAP) nor the compounds purified analogous to the *N*-acetylmuramyl-tripeptide (*N*-acetylmuramyl-L-alanyl-D-glutamic acid and *N*-acetylmuramyl-L-alanine) showed any effect on cell separation.

Effects of nalidixic acid and low concentrations of ampicillin on the chain-forming mutant D22 (*envA*). The addition of both nalidixic acid and ampicillin to growing chains of EnvA cells decreases the frequency of septa rapidly (15). The effect of these drugs on the septum process in the chain-forming mutant D22 was therefore studied. The generation time in broth for strain D22 (*envA*) was 30 min, and the average chain contained three septa. If formation of new septa was inhibited and the rate of completion of initiated ones was unaffected, the average chain would contain only one septum after 30 min. Moreover, by this time a doubling in cell number should be expected. This result was obtained after inhibition of septum initiation with nalidixic acid (Fig. 3). During the first 30 min the decrease in septa was paralleled by an increase in cell number, showing that the loss of a visible septum was due to a physical cell separation (Fig. 3). In comparison, ampicillin (5 μg/ml) resulted in a more rapid initial decrease in the frequency of septa per chain

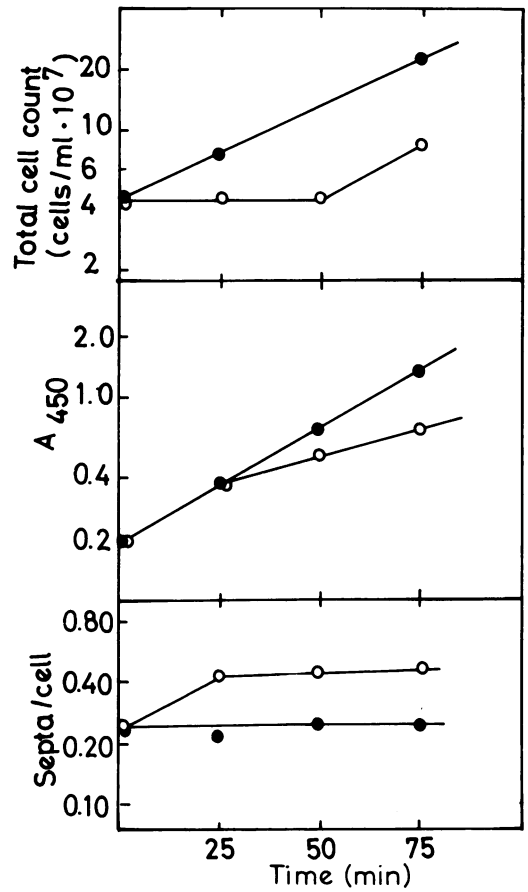

FIG. 1. *Effect of N-acetylmuramyl-L-alanyl-D-glutamyl-meso-DAP on septum separation in strain D21 (wild type). The strain was grown in LB medium. At zero time 100 μl of N-acetylmuramyl-tripeptide (400 nmol) was added to 500 μl of culture. In the control (100 μl) were taken into 100 μl of ice-cold 0.1% (100 μl) were taken into 100 μl of ice cold 0.1% formaldehyde. Cell number, absorbance, and the frequency of septa per cell were determined as described in Material and Methods. Symbols: ●, control; ○, control plus N-acetylmuramyl-tripeptide.*

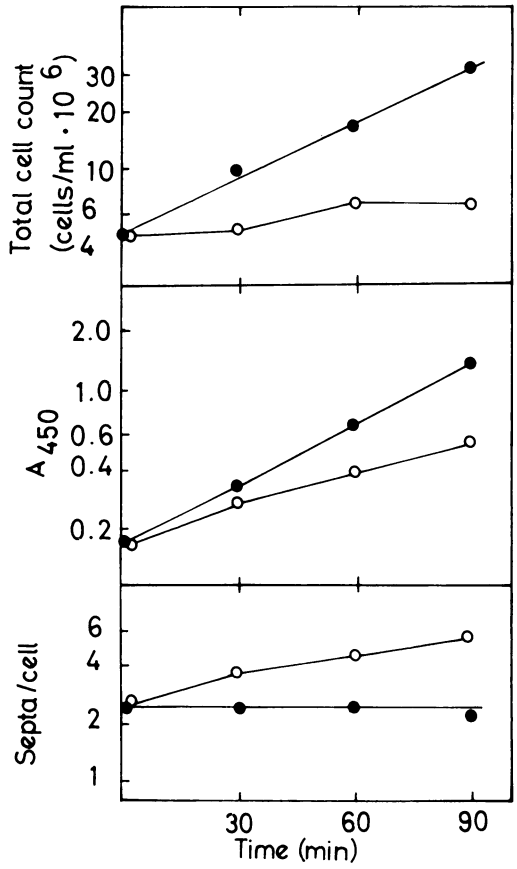

FIG. 2. *Effect of N-acetylmuramyl-L-alanyl-D-glutamyl-meso-DAP on septum separation in strain D22 (envA). The experimental conditions were as in Fig. 1. Symbols: ●, control; ○, control plus N-acetylmuramyl-tripeptide.*

(Fig. 3). Even here this decrease was initially followed by an increase in cell number. However, after 10 min of ampicillin treatment no further increase in cell number was recorded, despite a successive decrease of septa.

The filament-inducing agents, nalidixic acid and ampicillin, thus have a disparate effect on initiated septa. We, therefore, analyzed whether these agents affected the low amidase activity of exponentially growing cells of strain D22 (*envA*). Both agents caused a rapid and significant increase in amidase activity (Fig. 4).

DISCUSSION

In the mutant carrying the *envA* mutation, septal peptidoglycan is visualized as a single dense structure, the thickness of which reveals that it is composed of two fused layers (15). The association between the layers is probably covalent in nature, as a separation was not achieved even after 1 h of boiling in 4% sodium dodecyl sulfate. Therefore, septum formation and septum separation in strain D22 (*envA*) must involve enzyme activities that fuse and separate septal peptidoglycan. The stable septa in strain D22 (*envA*) could thereby be due to an increased fusing activity or to a decreased septal peptidoglycan-splitting activity. Our data favor the latter alternative and suggest a role for *N*-acetylmuramyl-L-alanine amidase in the septum separation process in *E. coli*.

To determine a role for the amidase, we have here studied: (i) the amidase level in the chain-forming mutant D22 (*envA*) and its wild type; (ii) the effects of various physiological treatments on the amidase activity; and (iii) the effect obtained by the addition of the assumed substrate. The following results were obtained. (i) Compared to the wild type, the mutant showed a sixfold reduction in amidase activity when the strains were grown in rich medium. (ii) Genetic as well as physiological suppression of chain formation was associated with an increase in amidase activity. (iii) Wild-type cells grown in the presence of PEA showed an increased frequency of septa and a decreased amidase activity. (iv) When the substrate *N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP was added to growing wild-type and mutant cells, an increase in the frequency of septa was observed.

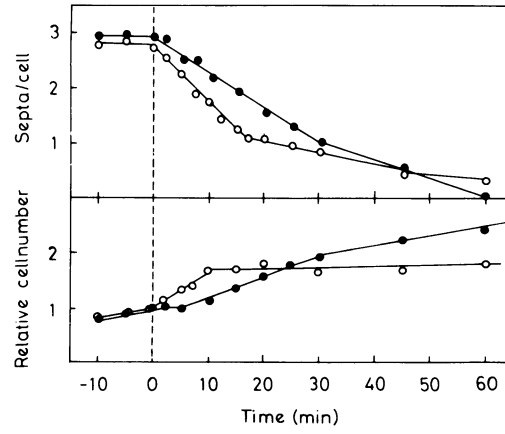

FIG. 3. *Effects of nalidixic acid and ampicillin on the frequency of septa and cell number in strain D22 (envA). Strain D22 was grown to a steady-state chain length in LB medium. At zero time nalidixic acid (20 μg/ml) or ampicillin (5 μg/ml) was added. The cell number and the frequency of septa per cell were determined as described in Material and Methods. Shallow constrictions were not counted as septa. Symbols: ●, culture to which nalidixic acid was added; ○, culture to which ampicillin was added.*

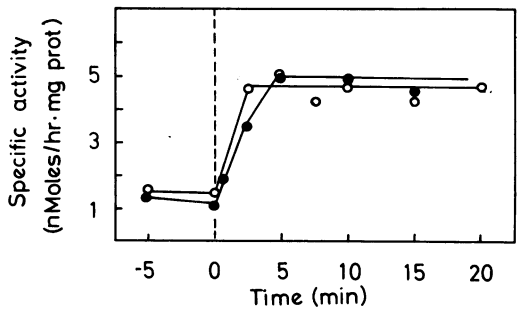

FIG. 4. *Effects of nalidixic acid (20 μg/ml) and ampicillin (5 μg/ml) on N-acetylmuramyl-L-alanine amidase activity in strain D22 (envA). Cells were grown in steady state in LB medium. At zero time nalidixic acid (●) or ampicillin (○) was added. Samples (100 ml) were withdrawn and rapidly chilled on ice (−20°C). Enzyme activities were determined as described in Material and Methods.*

This can be interpreted as a competitive in vivo inhibition of the amidase. Taken together these data indicate that the amidase is involved in the septum separation process of *E. coli* K-12.

Studies on *N*-acetylmuramyl-L-alanine amidase in *S. pneumoniae* have revealed that choline-containing lipoteichoic acids are required for enzyme activity (9). Replacement of choline for ethanolamine inhibited the amidase (9). Therefore, in *S. pneumoniae* lipoteichoic acids may exert a regulatory function on the amidase. A similar function for the teichoic acids of *B. subtilis* has recently been suggested (8). In this context the observed association of amidase to the outer membrane of *E. coli* is interesting. This association appears loose, as 50% of the total activity is apparently not membrane bound (22). The mutant D22 (*envA*) is affected in the outer membrane, leading to an increased penetrability (6, 14). The biochemical nature of this lesion is not known. However, the mutant D22 (*envA*) differs significantly from its wild type in the relative distribution of outer membrane phospholipids (16, 25). All physiological treatments suppressing the low amidase activity of *envA*, i.e., the addition of PEA, ampicillin, and nalidixic acid, also restore the barrier function of the outer membrane of cells carrying the *envA* mutation (15, 16). We have not been able to demonstrate a leakage of amidase out from the cells. Our data, therefore, indicate that the outer membrane exerts a regulatory function on the amidase activity. The low amidase activity in strain D22 (*envA*) could then be explained by a modified outer membrane structure.

Both ampicillin and nalidixic acid inhibit cell division (2, 20, 26). The latter drug interferes with deoxyribonucleic acid replication and acts at the level of septum initiation (2, 26). Ampicillin treatment of synchronous cells of *E. coli* B/r caused an inhibition of division when added at all stages in the cell cycle (2). Therefore, ampicillin apparently inhibits septum formation. In *E. coli* at least three enzymatic activities that are inhibited by penicillins have been identified: DD-alanine carboxypeptidase, endopeptidase, which is thought to be another manifestation of DD-carboxypeptidase, and the transpeptidase that catalyzes the cross-linking reaction (12). It has been suggested that the transpeptidase is involved in septum formation (7). When ampicillin was added to chains of EnvA cells, the initial decrease in septa was higher than that found with nalidixic acid and exceeded the normal rate of septum completion. No difference in amidase activity was found in ampicillin- and nalidixic acid-treated cells. It may, therefore, be suggested that ampicillin inhibits septal fusing, thereby increasing the rate of septum separation.

After 10 min of ampicillin treatment no further increase in cell number was recorded, despite a continuous decrease in the frequency of visible septa. This suggests that partial septa are not allowed to go to cell separation. The decrease in visible septal structures indicates that they are gradually transformed into shallow constrictions impossible to distinguish from the remaining part of the peripheral wall.

It is suggested that septal fusing and septal splitting activities are in a state of equilibrium in the developing septum. In the mutant D22 (*envA*) the splitting activity is reduced, causing an unbalance between these two activities, which in turn leads to chain formation.

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