Autolysis of a Division Mutant of *Escherichia coli*

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The pleiotropic character of the *envC* chain-forming mutant of *Escherichia coli* was found to include leakage of periplasmic enzymes and an abnormal tendency to autolyse. Washed suspensions of *envC* cells released murein fragments into the supernatant, and cell extracts from the mutant were richer than those of the wild type in exo- β -N-acetylglucosaminidase (187% of the wild-type value) and in soluble endopeptidase (256%) activities, but N-acetylmuramoylamidase, D,D-carboxypeptidase, L,D-carboxypeptidase and transglycosylase were not markedly different. When *envC* cells were grown in medium containing 0.58 M-sucrose, the chains broke up into rods, the L,D-carboxypeptidase activity increased about sixfold and D,D-carboxypeptidase 1B about twofold. It is suggested that L,D-carboxypeptidase is involved in septum splitting. The results suggest that the triggering of autolysis in *E. coli envC* depends on the alteration of envelope constituents rather than on an enhanced activity of murein hydrolases.

INTRODUCTION

Escherichia coli is very resistant to peptidoglycan breakdown by endogenous hydrolases and reports of autolysis of this species are rare. Cell suspensions of *E. coli* in growth medium or in buffer are fairly stable (Hartmann *et al.*, 1974), but chemical treatment and osmotic shock, individually (Leduc & van Heijenoort, 1980) or combined with nutritional shift (Mitchell & Moyle, 1956), can induce rapid autolysis. Autolytic enzymes are believed to participate in wall growth, division and shape maintenance of growing cells but their true physiological role is controversial (Fan, 1970; Ghuysen & Shockman, 1973; Fein & Rogers, 1976; Hinks *et al.*, 1978*a, b*).

We have reported earlier that growing cultures of the *envC* mutant of *E. coli* K12 contain a significant fraction of cells undergoing autolysis (Rodolakis *et al.*, 1973). The mutant grows in the form of long chains and electron microscopy shows that septum formation is often incomplete and irregular. The *envC* mutant is hypersensitive to various antibiotics, dyes and detergents (Starka *et al.*, 1974) and leaks the periplasmic enzymes alkaline phosphatase and ribonuclease during growth (Starkova *et al.*, 1978). These varied properties have prompted us to examine the autolytic behaviour of the *envC* mutant and the activity of several of its murein hydrolases.

METHODS

Bacterial strains, culture conditions and preparation of extracts. *Escherichia coli* K12 strain P678 and its isogenic, chain-forming *envC* derivative were used. Their genotypes and culture conditions were as previously described (Michel *et al.*, 1977). Cells were harvested in mid-exponential phase at an A_{450} of 0.5–0.6 (Bausch and Lomb Spectronic 21 instrument), centrifuged at 3000 g for 15 min at 4 °C, resuspended in 5 ml per g wet wt of

bacteria of cold 20 mM-potassium phosphate buffer pH 7.0 or 30 mM-Tris/HCl pH 7.3, broken in a French press (Aminco) at 4–5 ton in⁻² (62–77 MPa), and centrifuged at 3000 *g* for 10 min; the supernatant was recentrifuged at 100 000 *g* for 60 min at 4 °C. The resulting supernatant is referred to as the soluble fraction, and the pellet which was taken up in 1–2 ml of cold phosphate buffer or Tris/HCl buffer as the envelope fraction. Extracts were stored at –18 °C.

To measure autolytic activity in growing cells, we used a medium containing 100 mM-Tris/HCl pH 7.4, 0.2 mM-K₂HPO₄, 0.1% (w/v) NH₄Cl, 1 mM-MgSO₄, 0.5% (w/v) NaCl and 0.2% (w/v) glucose, supplemented with threonine (100 µg ml⁻¹), leucine (20 µg ml⁻¹), thiamin (1 µg ml⁻¹), and Casamino acids (100 µg ml⁻¹). β -Galactosidase was induced by adding 0.5 mM-isopropyl- β -D-thiogalactopyranoside (IPTG) about 1 h before harvesting.

Substrates. [¹⁴C]Diaminopimelate-labelled sacculi and [³H]diaminopimelate-labelled bis(disaccharide-tetrapeptide) were prepared from cell walls of *E. coli* W7 *lysA dap* by methods already described (Primosigh *et al.*, 1961; Hartmann, 1973; Hartmann *et al.*, 1974). *N*-Acetylmuramoyl-L-alanyl- γ -D-glutamyl-L-[³H]*meso*-diaminopimelic acid was kindly provided by J. van Heijenoort.

UDP-*N*-acetylmuramoyl-L-alanyl- γ -D-glutamyl-(L)-*meso*-diaminopimelic acid (UDP-MurNAC-tripeptide) was extracted from *Bacillus cereus* T (received from J. van Heijenoort) by a modification of the method described by Lugtenberg *et al.* (1972); cells grown in complete medium were harvested in exponential phase and incubated for 90 min in half the volume of synthetic medium containing 150 µg *meso*-diaminopimelic acid ml⁻¹ and 100 µg D-cycloserine ml⁻¹ (Strominger *et al.*, 1959). The extraction and purification of the precursor were performed without modification.

UDP-*N*-acetylmuramoyl-L-alanyl- γ -D-glutamyl-(L)-*meso*-2,6-diaminopimelyl-D-[1-¹⁴C]alanyl-D-[1-¹⁴C]alanine (UDP-MurNAC-pentapeptide) was prepared by incubating the UDP-MurNAC-tripeptide with DL-[1-¹⁴C]alanine (4.5 mCi mmol⁻¹; 166 MBq mmol⁻¹) and an extract of sporulating *Bacillus sphaericus* 9602 (provided by D. J. Tipper) as described by Guinand *et al.* (1974). This preparation racemizes alanine, synthesizes the dipeptide D-Ala-D-Ala and adds it to the tripeptide in one step.

UDP-*N*-acetylmuramoyl-L-alanyl- γ -D-glutamyl-(L)-*meso*-2,6-diaminopimelyl-D-[¹⁴C]alanine (UDP-MurNAC-tetrapeptide) was obtained by excision of the terminal alanine of the pentapeptide derivative using the exocellular D,D-carboxypeptidase of *Actinomadura* R39 (provided by J.-M. Ghuysen), prepared and purified through the first step as described by Leyh-Bouille *et al.* (1972). The conditions of incubation and separation of the reaction products by high-voltage electrophoresis have been described (Pellon & Michel, 1979). The yield was close to 95% and the specific activity of the UDP-MurNAC-tetrapeptide was 110 Bq nmol⁻¹.

UDP-MurNAC-L-Ala- γ -D-Glu-L-lysine was prepared by the method of Strominger *et al.* (1959) from *Staphylococcus aureus* incubated with D-cycloserine. The pentapeptide derivative was prepared exactly like the diaminopimelate-containing pentapeptide.

p-Nitrophenyl-*N*-acetyl- β -D-glucosaminide (*p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside) was from Sigma. DL-[1-¹⁴C]Alanine and 2,6-diamino[1,7-¹⁴C]pimelic acid were from the Commissariat à l'Energie Atomique, Saclay, France; 2,6-diamino[G-³H]pimelic acid dihydrochloride was from Amersham, U.K.

Enzyme assays. *N*-Acetylmuramoyl-L-alanine amidase (MurNAC-L-Ala amidase; EC 3.5.1.28) was assayed as described by van Heijenoort *et al.* (1975). Endopeptidase was assayed by the method of Tamura *et al.* (1976) except that the reaction was stopped by boiling for 5 min. The reaction products were located on the chromatogram by counting 0.5 cm sections of each strip. Activities were expressed as percentage hydrolysis min⁻¹ (mg protein)⁻¹. β -*N*-Acetyl-D-glucosaminidase (EC 3.2.1.30) was assayed by the method of Yem & Wu (1976). D,D-Carboxypeptidase and L,D-carboxypeptidase activities were assayed according to Tamura *et al.* (1976) and transglycosylase was assayed according to Mett *et al.* (1980); 1 nmol of the diaminopimelate substrate or 0.69 nmol of the lysine substrate was used. β -D-Galactosidase (EC 3.2.1.23) and alkaline phosphatase (EC 3.1.3.1) were assayed by the methods described by Wallenfels (1962) and Garen & Levinthal (1960), respectively.

Analytical methods. Murein components were measured as described by Ghuysen *et al.* (1966); murein cross-linkage was measured by the method of Braun & Wolff (1975), 3-deoxy-D-manno-octulosonic acid (KDO) by the method of Osborn *et al.* (1972), and protein by the Lowry method.

Autolysis of washed cells. Bacteria growing exponentially in complete medium with IPTG (Michel *et al.*, 1977) were washed in 0.05 M-Tris/maleate buffer pH 7.0 and resuspended in buffers pH 6.0 to 8.4 (0.05 M-Tris/maleate) or pH 8.4 to 9.4 (0.05 M-glycine/NaOH). The suspensions were incubated at 37 °C for 2 h and the *A*₄₅₀ and β -galactosidase activity in the supernatant were determined.

RESULTS AND DISCUSSION

Autolysis and leakage of cellular components

In preliminary experiments we observed that the absorbance and viable cell count of *envC* cultures grown in agitated complete or minimal medium decreased 2 h after the stationary

Table 1. Comparison of amounts of cellular components in culture fluids of wild-type (*envC*⁺) and *envC* strains of *E. coli*

The numbers represent the percentage of the total amount (cells plus culture fluid) for each component.

Cell component	<i>envC</i> ⁺		<i>envC</i>	
	Exponential phase	Stationary phase	Exponential phase	Stationary phase
Alkaline phosphatase	0	9.7	10.8	38
β -Galactosidase	1	1.6	4	14
5'-Nucleotidase	ND	ND	29	75
Protein	1.5	1.2	9	11
KDO	1	3	19	45

ND, Not determined.

Table 2. Release of β -galactosidase by non-growing *envC*⁺ and *envC* strains of *E. coli*

Cells washed in 0.05 M-Tris/maleate buffer pH 7 were suspended in 0.05 M-Tris/maleate buffer (samples 1 to 6) or 0.05 M-glycine/NaOH buffer (samples 7 to 9) and incubated for 120 min at 37 °C. The A_{450} was then read, the cells were centrifuged and β -galactosidase activity was measured in the supernatant. β -Galactosidase activity is expressed in arbitrary units. ΔA_{450} represents the percentage decrease in absorbance. The initial A_{450} was 0.6 in all the samples.

Sample no.	pH	<i>envC</i> ⁺		<i>envC</i>	
		β -Galactosidase	ΔA_{450}	β -Galactosidase	ΔA_{450}
1	6.0	0	9	33	11
2	6.4	0	10	22.5	14
3	7.0	0	10	31	20
4	7.4	0	10	32	20
5	8.0	5	11	35	22.5
6	8.4	9	22	51.5	35.5
7	8.4	0	11	165	29.5
8	9.0	0	19.5	222	46.5
9	9.4	9	26	231	51

phase was reached. The decrease was about 20% for the A_{450} and 25% for the viable count. The parent strain was much more stable under the same conditions, and a slow decrease of both parameters started only after 24 h incubation. Electron microscopy of stationary-phase *envC* cells showed a significant proportion of lysed cells. It was reported previously that growing *envC* cultures release alkaline phosphatase without osmotic shock and it was suggested that the liberation of that periplasmic enzyme was the consequence of leakiness of the outer membrane (Starkova *et al.*, 1978). Thus, it was of interest to determine how much of this alkaline phosphatase release was the result of cell autolysis. The results in Table 1 indicate the amounts of various cell components found in the culture medium of *envC* and wild-type (*envC*⁺) bacteria. In exponentially growing cultures, *envC* lost 10% of its alkaline phosphatase, about 30% of its 5'-nucleotidase and 19% of its KDO [representing lipopolysaccharide (LPS) from the outer membrane]. Only 4% of the β -galactosidase was found in the medium, indicating indirectly that only a fraction of leaky cells had undergone autolysis. Leakage of periplasmic enzymes is not entirely unexpected in a strain that is abnormally permeable and sensitive to lysozyme without EDTA being present. It should be noted that the percentage of total KDO found in the culture medium of *envC* was greater than that of total β -galactosidase. This indicates that intact *envC* cells also shed LPS from the cell envelope. With the exception of total protein, the supernatant of stationary-phase *envC* cultures contained about three times as much of the released compounds as the supernatant of exponentially growing cultures. Exponentially growing *envC*⁺ cells, on the other hand,

Table 3. *Relative specific activities of murein hydrolases in the soluble and envelope fractions of envC⁺ and envC strains of E. coli grown in the presence or absence of 0.58 M-sucrose*

The values are expressed as a percentage of those obtained with the *envC⁺* strain grown in normal medium.

Enzyme	Cell fraction	<i>envC⁺</i>		<i>envC</i>	
		Normal medium	Medium + sucrose	Normal medium	Medium + sucrose
<i>N</i> -Acetylglucosaminidase	Soluble	100	157	187	177
	Envelope	100	177	147	216
<i>N</i> -Acetylmuramoylamidase	Soluble	100	110	88	116
D,D-Carboxypeptidase 1A + 1B*	Envelope	100	89	128	287
D,D-Carboxypeptidase 1A†	Envelope	100	ND	138	87
L,D-Carboxypeptidase	Envelope	100	100	66	450
Endopeptidase	Soluble	100	83	256	102
	Envelope	100	77	91	254
Transglycosylase	Envelope	100	166	157	216

ND, Not determined.

* Assayed with UDP-MurNAc-pentapeptide containing diaminopimelic acid (see text).

† Assayed with UDP-MurNAc-pentapeptide containing lysine instead of diaminopimelic acid (see text).

released barely detectable amounts of cellular components. Stationary-phase *envC⁺* cultures liberated about 10% of their total alkaline phosphatase but very little of other components.

After being washed, *envC* cells underwent even more pronounced autolysis. The results in Table 2 show that they released a rather constant amount of β -galactosidase at pH values between 6 and 8. Above pH 8 the breakdown of cell integrity rose steeply. The release of β -galactosidase roughly corresponded to the degree of loss of absorbance of the cell suspension. The release process was greatly influenced by the buffer constituents. For example, the activity of liberated β -galactosidase was three times higher in glycine buffer at pH 8.4 than in Tris/maleate buffer of the same pH. No significant enzyme release from wild-type cells was observed at any pH in glycine or in Tris/maleate buffers.

The supernatant of *envC* cells incubated as above in 0.05 M-Tris/maleate buffer pH 8.5 contained murein-like fragments insoluble in absolute alcohol, but soluble in 66% (v/v) ethanol, giving five ninhydrin-positive spots upon chromatography as described by Primosigh *et al.* (1961). All these spots, upon hydrolysis and chromatography, were shown to contain some or all of the murein components (hexosamine, alanine, glutamic acid) and three of them contained diaminopimelic acid. It seems most likely that they represent murein degradation products. None of these compounds was detected in supernatants of similarly treated wild-type cells (results not shown).

Despite a strong tendency to autolyse, the *envC* mutant seemed to have a normal rate of murein synthesis as measured by incorporation of labelled diaminopimelic acid during exponential growth. Furthermore, the murein has a normal composition and degree of cross-linkage (D. Karibian, unpublished results).

Murein hydrolase activities in envC cells

The susceptibility of *envC* cells to autolysis could imply poorly controlled activity, or abnormal levels, of murein hydrolases. We therefore compared the activities of six of these enzymes in *envC* and wild-type cells. It was reported earlier that the anomalous division and chain formation of the *envC* mutant can be phenotypically corrected by adding 0.58 M-sucrose to the medium (Starka *et al.*, 1974). Therefore, murein hydrolase activities were measured in extracts of cells grown in the presence or absence of sucrose (Table 3). The

Table 4. Influence of lysophosphatidylethanolamine (LPE), Triton X-100 and sucrose on murein hydrolase activities of *envC*⁺ and *envC* strains of *E. coli*

The enzyme activities are expressed as a percentage of those obtained with the *envC*⁺ strain with no addition to the reaction mixture.

Addition	L,D-Carboxypeptidase		Endopeptidase		N-Acetylglucosaminidase		Transglycosylase	
	<i>envC</i> ⁺	<i>envC</i>	<i>envC</i> ⁺	<i>envC</i>	<i>envC</i> ⁺	<i>envC</i>	<i>envC</i> ⁺	<i>envC</i>
None	100	80	100	115	100	129	100	ND
LPE*	117	102	88	95	103	122	102	ND
Triton	96	104	126	140	104	124	ND	ND
X-100 (0.2%)								
Sucrose (0.58 M)	100	98	ND	ND	ND	ND	98	ND

ND, Not determined.

* LPE (1.8 µmol) was mixed with extract before adding other solutions, to give final concentrations of 72 mM, 46 mM, 5 mM and 18 mM in the L,D-carboxypeptidase, endopeptidase, N-acetylglucosaminidase and transglycosylase reaction mixtures, respectively.

soluble fraction of *envC* was richer than that of the wild type in N-acetylglucosaminidase and endopeptidase activities. It should be noted that the hydrolysis of *p*-nitrophenylglucosaminide measures only exoglucosaminidase activity, which is not known to be involved directly in autolysis. The transglycosylase activity in the soluble fractions was low in both strains (not shown) and represented only a small fraction of the activity found in the envelopes. The activities of other enzymes did not seem to be markedly different between the strains. The fact that the functions and control of murein hydrolases *in vivo* are obscure makes it difficult to estimate whether or not differences of 20–50% are significant. Although Kitano *et al.* (1980) were able to attribute the autolysis resistance of β -lactam-tolerant *E. coli* strains to a deficiency in N-acetylmuramidase (transglycosylase) activity, it would be less justifiable to attribute the autolysis sensitivity of the *envC* strain to any one of the several murein hydrolases that are in excess. Indeed, Yem & Wu (1976) showed that mutants of *E. coli* with less than 0.2% of the wild-type level of exo- β -N-acetylglucosaminidase activity grew and divided normally in rich or minimal media, and their morphology was also normal. On the other hand, MurNAc-L-Ala amidase-deficient and endo- β -N-acetylglucosaminidase-deficient mutants of *Bacillus subtilis* formed very long chains of unseparated cells (Fein & Rogers, 1976).

What is more intriguing is that when *envC* is grown in medium containing 0.58 M-sucrose, a condition that causes the chains of cells to break up into rods, the L,D-carboxypeptidase activity is about sixfold higher than in cells grown without supplementary sucrose (Table 3). Beck & Park (1976) showed that the amount of this enzyme increases just before cell division, suggesting that the L,D-carboxypeptidase might be involved in cell separation. However, the sucrose effect seemed to be on enzyme synthesis rather than on enzyme activity, since the same concentration of sugar added to an enzyme reaction mixture had no effect (Table 4). The D,D-carboxypeptidase activity of sucrose-grown *envC* was also higher than normal, but the increase was less marked (Table 3).

However, when UDP-MurNAc-pentapeptide containing lysine instead of diaminopimelate was used as the substrate of D,D-carboxypeptidase, the activity in the extract of sucrose-grown *envC* showed no increase at all. The lysine-containing substrate allows a distinction to be made between the two D,D-carboxypeptidases, 1A and 1B, shown to exist by Tamura *et al.* (1976), since it is used only by the 1A enzyme. Thus, the 1A enzyme, which alone was measured with the lysine-containing substrate, is not increased in sucrose-grown *envC*. The increased activity observed with the natural substrate was therefore due only to the 1B enzyme. The quantitative interpretation of these results is further complicated by the fact that the product of the reaction is the substrate of L,D-carboxypeptidase, which, being very

active in these cells, would tend to increase the apparent D,D-carboxypeptidase activity. However, all the activities represent initial catalytic rates and therefore might not be greatly affected by this side-reaction. The latter problem can and will soon be overcome by using substrate labelled only in the terminal alanine (Gorecki *et al.*, 1975).

A recent report from this laboratory showed that the *envC* membranes contained appreciable levels of lysophosphatidylethanolamine (LPE) as a result of abnormal phospholipase A activity (Michel & Starka, 1979). Since lysophospholipids and synthetic detergents have been shown to influence many membrane-associated enzymes (Rothfield & Romeo, 1971), it was of interest to test the effect of LPE on murein hydrolase activities. The results in Table 4 show that LPE as well as the non-ionic detergent Triton X-100 did not significantly influence the activities tested. Therefore, if the fragility of *envC* cells is due to the presence of lysophospholipid in the envelope, the primary effect is probably not on murein hydrolase activities.

The correlation between cell wall growth, septum formation and murein hydrolase activities remains unclear. For instance, it was reported recently that the mutants of *E. coli* deficient in D,D-carboxypeptidase 1A (*dacA*) or in D,D-carboxypeptidase 1B (*dacB*) grow and synthesize murein normally (Matsushashi *et al.*, 1978). This would mean that these two murein hydrolases do not participate either in murein synthesis or in septum splitting. This latter function was attributed by Wolf-Watz & Normark (1976) to the MurNAc-L-Ala amidase. Their conclusion was based on the fact that the *envA* chain-forming mutant had a sixfold lower amidase activity than the parent *E. coli* D21. However, the present results show that in spite of its chain-forming phenotype, *envC* has the same amidase activity as the parent and that this activity does not change in *envC* cells grown in the presence of 0.58 M-sucrose, a condition under which they divide at the same rate as the parent strain. On the other hand, L,D-carboxypeptidase, which was lower in *envC* than in the wild type, increased considerably when the mutant was grown in the presence of hypertonic sucrose. This suggests that L,D-carboxypeptidase is involved in septum splitting, but the problem merits further investigation.

It is most probable that the murein hydrolase activities measured in disrupted cells do not correspond to the actual activities needed for normal growth and division. In the case of *envC*, its autolytic behaviour can hardly be explained by the observed increases in endopeptidase and *N*-acetylglucosaminidase activities in cell fractions. In the light of these considerations, it seems probable that the triggering of autolysis in *E. coli* depends rather on the state and conditions of other envelope constituents than on the activity of murein hydrolases. Previous results from this laboratory showing important alterations of the *envC* outer membrane are not inconsistent with this view.

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