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Peptidoglycan hydrolases in an autolytic mutant of *Salmonella typhimurium*

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(2 figures)

Two peptidoglycan hydrolases were isolated from the autolytic mutant *Salmonella typhimurium* DA361 (*envD*). One of them, resistant to penicillin, was found free in the supernatant of partially purified envelopes sedimented by ultracentrifugation, and the other bound to the envelopes proved to be sensitive to the antibiotic. Both were able to hydrolyse *in vitro* high molecular weight non-specific peptidoglycan isolated from *E. coli* W7 labelled with [^{14}C]diaminopimelic acid. Similar enzymatic activities were separated also from *S. typhimurium* DA362 (*envD*^{*}) a non-lytic isogenic pair of the above and from the wild type strain LT-2. All of the hydrolytic activities reported here were strongly inhibited when DNA was added to the assay systems. The peptidoglycan hydrolases isolated from the autolytic mutant suffered a competitive inhibition while those from the non-lytic strains were apparently inhibited in uncompetitive modal relationship. It is postulated that the inhibitory effect may bear affinity with the preservation of DNA sites of attachment to cell membranes sustaining peptidoglycan structure and functions.

Introduction

Spontaneous autolysis is a characteristic feature of many Gram positive strains and this feature was thoroughly studied (SHOCKMAN, 1965; GHUYSEN & SHOCKMAN, 1973; JOSEPH & SHOCKMAN, 1976). Enterobacteria do not harbor such property and therefore do not undergo spontaneous autolysis at high rate unless some kind of inductor is applied to them. ANTÓN & ORCE (1976) reported the isolation of a series of autolytic mutants characterized by the loss of 80-90% of their cellular integrity shortly after reaching stationary phase, when grown in nutrient broth at 37 °C. These mutants presented also increased sensitivity to sodium dodecyl sulphate, deoxycholate and EDTA, and also a morphological tendency to form chains of short rods. The above mentioned authors demonstrated the responsibility for the complex abnormal phenotype of a single stable mutation, suggesting the observed anomalies as pleiotropic effects of a single gene mutation, called *envD*.

PIZARRO *et al.* (1985) reported that the autolytic activity of one of such mutants, *Salmonella typhimurium* DA361 (*envD*) was accompanied by a 100% increased

content of diphosphatidylglycerol (DPG), when compared with an isogenic non-lytic strain *S. typhimurium* DA362 (*envD*⁺) or the wild type LT-2. They showed also that environmental conditions known to influence membrane activities, like the presence of ethanol during growth, led to the biochemical and phenotypical correction of most of the abnormalities displayed by the autolytic strain DA361. The unusual phenotype of this mutant point to generic alterations produced at the envelope level by a single gene mutation, leading to changes in permeability and/or regulation of membrane functions.

Based on the consideration that peptidoglycan hydrolases may play a key role in the lytic process, we assumed that the spontaneous autolysis of the *envD* mutant could be related with changes in the regulation mechanism set on by biochemically altered envelopes and, therefore, we undertook the study of hydrolases obtained from the mutants. We report here the isolation, partial purification and characterization of two peptidoglycan hydrolases obtained from both, DA361 and DA362 strains, which were undistinguished with respect to their origin, except for the kinetics of the inhibition induced by the presence of DNA in the assay system, which was competitive in the autolytic strain and uncompetitive in the isogenic non-lytic strain. The peptidoglycan hydrolases specific activities, their biochemical properties and significance in the autolytic process are discussed in the text.

Materials and Methods

Strains and growth conditions. *S. typhimurium* DA361 and DA362 (ANTON & ORCE, 1976), *S. typhimurium* LT-2 and *E. coli* W7 (*dap*⁻, *lys*⁻) were grown in nutrient broth (Difco) at 37 °C with shaking.

Envelopes obtention. Partially purified envelopes were obtained from DA361 (*envD*) and DA362 (*envD*⁺) strains grown in 1 litre volumes to an optical density (OD) of about 0.700 at 650 nm. Cells harvested by centrifugation (15 min at 4 000 rev./min) were resuspended in 40 ml of cold 10 mM Tris-maleate buffer (pH 6.5) and disrupted by a three times passage through an *Aminco* French Pressure Cell. Remaining whole cells and debris were eliminated at low speed in a refrigerated centrifuge and the envelopes were sedimented at 100 000 × *g* in a *Spinco-Beckman* ultracentrifuge.

Autolysis. Envelope suspensions in 50 mM Tris-HCl buffer (pH 8.5) containing 10 mg/ml of protein and showing an absorbance of about 1 000 at 650 nm were incubated at 37 °C in a thermostatically controlled *Beckman* spectrophotometer. The autolysis was measured by the decrease in OD_{650 nm} as a function of time.

Obtention of peptidoglycan hydrolases. Two peptidoglycan hydrolases able to degrade *in vitro* [¹⁴C]peptidoglycan were found in the above envelope suspensions : (i) A soluble hydrolase (protein content : 2 mg/ml), found free in the supernatant of envelopes sedimented by ultracentrifugation. This crude extract was purified by fractionated ammonium sulphate precipitation (40-60%), resulting in a 400-fold increased specific activity. (ii) The second hydrolytic activity was found bound to the pelleted envelopes and was isolated by the procedure of HARTMANN *et al.* (1972) with some modifications. Sedimented envelopes were solubilized at room temperature in a mixture of 2% Triton X-100, 0.5 mM NaCl and 10 mM Tris-maleate (pH 6.6). The mixture was centrifuged 30 min at 85 000 × *g* and then concentrated by ultrafiltration (protein content : 0.3 mg/ml).

Obtention of radioactive peptidoglycan. [^{14}C]-labelled peptidoglycan sacculi obtained from *E. coli* W7 were used as substrate in the assay of peptidoglycan hydrolases. Cells were grown in nutrient broth (6 litres) added with 2 $\mu\text{g}/\text{ml}$ of cold diaminopimelic acid plus 0.1 $\mu\text{Ci}/\text{ml}$ of meso-2-6-diamino [^{14}C]pimelic acid, specific activity 300-350 mCi/mmol. At an OD of about 0.800 cells were harvested by centrifugation and radioactive sacculi isolated following the procedure of VERWER *et al.* (1978).

Peptidoglycan hydrolases assay system. The enzymatic activities of isolated peptidoglycan hydrolases were assayed in an incubation mixture containing : 10 μl of 1 M acetate buffer (pH 4.5), 100 μl of the corresponding peptidoglycan hydrolase and after a 10 min-incubation at 37 °C, 100 μl of [^{14}C]-labelled peptidoglycan (10 000 cpm) were added and the whole was incubated 90 min at 37 °C. The reaction was stopped with 2 ml of 10% trichloroacetic acid, left 10 min in an ice bath and the precipitate pelleted in a refrigerated centrifuge (10 000 $\times g$, 15 min). 0.5 ml of the supernatant was transferred to vials containing 5 ml of Bray's scintillation fluid and the radioactivity counted in a Beckman T-3150 scintillation counter. In typical experiments 20-30% of the label was found free in the supernatant.

Inhibitor studies. The presence of an inhibitory factor in the free-peptidoglycan hydrolase was inferred when finding that it could be purified 400-fold, and confirmed when showing that aliquots pretreated 10 min at 60 °C inhibited the reaction of the purified free-peptidoglycan hydrolase and also that bound to envelopes. DNA was identified as a factor responsible for the inhibitory effect by treating the crude extract (i) as follows : a) by boiling it 10 min in a water bath, and b) by digesting untreated portions with 50 $\mu\text{g}/\text{ml}$ of either pronase, DNAase or RNAase (30 min at 37 °C) and then boiling the mixtures during 5 min. Precipitates formed during these treatments were eliminated by 20-min centrifugation at 12 000 $\times g$. When required 50 μl of clear supernatant of each of the individual extracts were added to the assay system.

Proteins determination. Proteins were measured by the procedure of LOWRY *et al.* (1951) or by the modification of WANG & SMITH (1975) for samples in the presence of Triton X-100. Bovine serum albumin was used as a standard.

Chemicals. All reagents were analytical grade. Radioisotopes were purchased from Commissariat à l'Energie Atomique, Paris, France.

Results

S. typhimurium DA361 grown in nutrient broth at 37 °C lyses spontaneously shortly after entering stationary phase, at the point where culture's pH became alkaline. Cells from any stage of growth autolyse at the same rate when washed free of nutrient medium and incubated at 37 °C in alkaline buffers (ANTÓN & ORCE, 1976).

We found that cell-free envelopes of the above strain retained the autolytic ability that characterizes whole cells. This is a thermolabile property as 75% of the lytic ability was destroyed after a 10-min heating at 60 °C (Fig. 1). The pH and temperature dependence were indicative of an enzymatic process and this led us to attempt the isolation of fraction(s) liable for the hydrolytic activities.

S. typhimurium DA361 cell-free extracts obtained as described in Materials and Methods, yielded two peptidoglycan hydrolases capable to degrade [^{14}C]-labelled peptidoglycan. One of the hydrolases was found free in the supernatant of envelopes

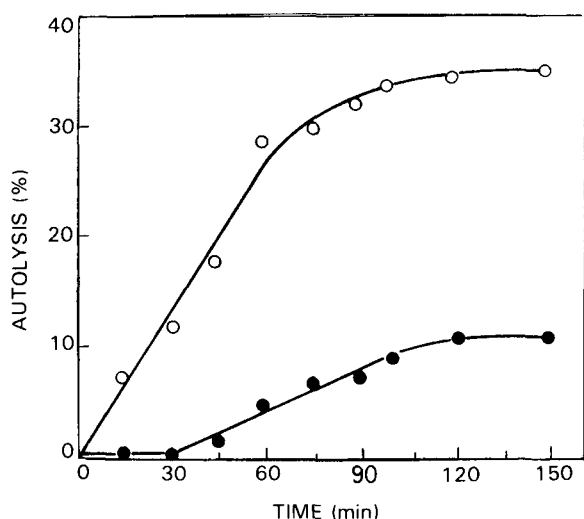


FIG. 1. Effect of preincubation temperature in the autolysis of *S. typhimurium* DA361 cell-free envelopes. Envelopes obtained as indicated above were resuspended in 100 mM buffer phosphates (pH 7.0), incubated 10 min at 60 °C (●) or at 0 °C (○) and then sedimented by ultracentrifugation. Autolysis was measured at 37 °C, by following OD decrease of envelopes suspensions in 50 mM Tris-HCl buffer (pH 8.5) containing 10 mg/ml total protein (see Materials and Methods).

sedimented by ultracentrifugation and the other, bound to envelopes, required extraction with Triton X-100 in 0.5 mM NaCl. The hydrolytic activities were assayed in the sensitive system developed by HARTMANN *et al.* (1972), using as substrate *E. coli* W7 [¹⁴C]peptidoglycan whose covalently linked lipoprotein makes undegraded substrate precipitable with trichloroacetic acid.

Both enzymatic activities reacted similarly when assayed with several effectors, except for one difference : the envelope-bound peptidoglycan hydrolase was inactivated

TABLE 1. Action of different effectors on the activity of free and envelope-bound peptidoglycan hydrolases from DA361.

Effector added to the assay system	conc. (mM)	Relative activity in peptidoglycan hydrolases :	
		Free	Bound
None	0	100	100
Mg ²⁺	3	120	104
Ca ²⁺	3	140	109
EDTA	3	104	84
SDS	3	0	1
Cysteine	3	130	104
<i>p</i> -Cl-mercuribenzoate	3	124	88
Penicillin G	200 U/ml	124	3

Enzymatic activities were assayed as described in Materials and Methods. The components were mixed and preincubated 10 min at 37 °C before [¹⁴C]-labelled peptidoglycan was added as substrate.

by 200 U/ml of penicillin, while the free hydrolase was resistant to the antibiotic (Table I). Equivalent peptidoglycan hydrolases were isolated from the non-lytic strain DA362 (*envD*⁺). All of them were thermosensitive since their hydrolytic ability was lost after 10-min heating at 60 °C (data not shown). By fractionated precipitation with (NH₄)₂SO₄ the specific activity of the soluble peptidoglycan hydrolase (i) was increased 400-fold and such high purification rate was assumed indicative for the presence in the crude extract of an inhibitor factor, whose nature was investigated in further experiments. For that purpose, aliquots of the crude extract (i) were inactivated by a 10-min incubation at 60 °C and then used to supplement the test system of HARTMANN *et al.* (1972). It was found that crude extract (i) heated 10 min at 60 °C arrested 80% of the lytic ability of either cell-free or envelope-bound hydrolases (Table II).

TABLE II. *Inhibitory effect of envelope-free crude extract (i) in the enzymatic activity of envelope-bound peptidoglycan hydrolases from DA361 and DA362.*

Addition to the assay system	Relative specific activity	
	DA361	DA362
None	100	100
Crude extract (i)	19	21
(i) + 10 min at 100 °C	14	20
(i) + Pronase	8	7
(i) + RNAase	102	63
(i) + DNAase	127	92
(i) + DNAase + RNAase	129	107
DNA (0.1 µg/ml)	13	10
RNA (0.1 µg/ml)	64	66

Aliquots of crude extract (i) obtained and pre-treated as indicated in Materials and Methods were added to the assay system. Yeast RNA was previously heated 10 min at 80 °C. Preincubation and substrate addition as in Table I.

To determine the inhibitor essential character, the mentioned heated crude extract was submitted to enzymatic attack with pronase, DNAase and RNAase. Our results indicate DNA as responsible of the inhibition, since 90% of the prevented hydrolytic capacity was restored after the heated extract was digested with DNAase, prior to its addition to the test system. Although to a lesser extent, RNAase was also efficient to counteract the inhibitory effect while proteolytic enzymes induced no changes in the results (Table II). The polynucleotide nature of the inhibitor was confirmed when finding that the addition of 0.1 µg/ml calf thymus DNA to the reaction mixture inhibited more than 80% of the lytic ability of the envelope-bound enzymes from either DA361 or DA362. A similar effect although to a much lower extent was found after 0.1 µg/ml yeast RNA was added to the test system (Table II).

The modality of prevention of the normal complexing of the hydrolases to radiolabelled substrate determined by DNA, was investigated in experiments where the effect of substrate concentration $[S]$ was measured against the velocity of reaction (V). As seen in Figure 2A the activity of the peptidoglycan hydrolase extracted from the autolytic strain DA361, when plotted as $1/V$ vs. $1/[S]$, showed that the slopes of inhibited and non-inhibited reactions intercept at the ordinates as is

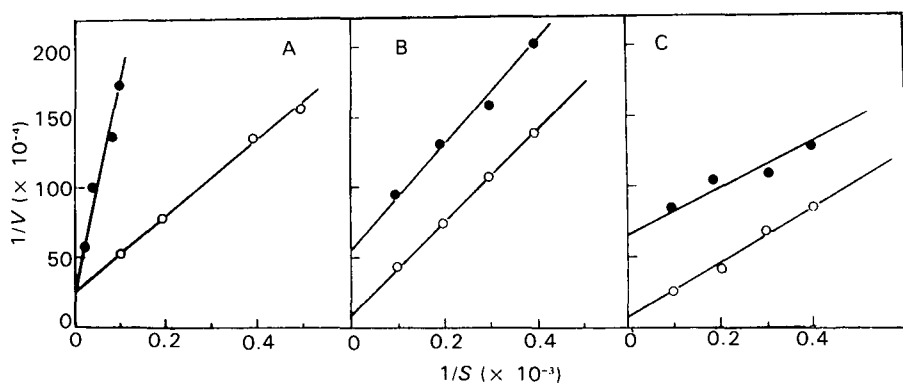


FIG. 2. Double reciprocal plot of envelope-bound peptidoglycan hydrolases from DA361 (A), DA362 (B), and LT-2 (C) in the presence (•) or absence (○) of the inhibitor (0.1 μ g of DNA), using [14 C]-labelled peptidoglycan as substrate (from 0 to 15 000 cpm/tube). The hydrolytic activities were assayed as described in Materials and Methods, except that reagents were incubated 60 min.

characteristic in reactions where competitive inhibition is effective. On the contrary, similar plotting of peptidoglycan hydrolytic activities isolated from the non-lytic strain DA362 or the wild type parental strain LT-2, showed apparently parallel slopes for reactions where the inhibitor was either present or absent, typical feature of uncompetitive inhibition (Fig. 2 B, C).

Discussion

Autolysis is not a distinctive quality of Gram negative and these organisms do not suffer spontaneous lysis unless induced by agents such as antibiotics, osmotic shock or chaotropic agents (KITANO & TOMASZ, 1979; BERENGUER *et al.* 1982; LEDUC *et al.* 1982; INGRAM, 1981). In every case autolysis show a marked dependence on temperature, pH or other factors that characterize enzymatic processes.

The isolation of *Salmonella typhimurium* mutants undergoing spontaneous autolysis (ANTÓN & ORCE, 1976), led us to study the biochemical and enzymatic properties of two peptidoglycan hydrolytic activities isolated from one of such mutants, the *envD* strain DA361. Peptidoglycan hydrolases showing similar properties were later extracted from DA362 an *envD*⁺ non-lytic mutant, and also from the wild type LT-2. They were all able to degrade [14 C]-labelled high molecular weight peptidoglycan sacculi into small radioactive products, in an enzymatic reaction inhibited by DNA. In fact, the inhibitory effect of polynucleotides in cell wall enzymes was described earlier in *E. coli* by TOMIOKA & MATSUHASHI (1978), and we consider worthwhile mentioning that the so far unreported *S. typhimurium* envelope-bound peptidoglycan hydrolase described here is also sensitive to penicillin, a well known inhibitor of cross-linking enzymes.

The study of the kinetics of polynucleotides inhibitory effect showed that the inhibition exerted by DNA is dependent on substrate concentration for hydrolases obtained from the autolytic DA361 mutant, while in either the non-lytic DA362 or the wild type LT-2 is apparently independent of such factor. Although no direct evidence was obtained to relate this difference with autolysis, we suggest that the

physiological function of the reported DNA inhibitory effect may be related to be sites of attachment linking DNA to the bacterial envelope *i.e.* cytoplasmic membrane-peptidoglycan-outer membrane (JACOB *et al.*, 1963; BAYER, 1968).

Moreover, we suggest that the discordance in the type of DNA inhibition may be connected with the alteration in the phospholipidic content of DA361 reported by PIZARRO *et al.* (1985), perhaps integrating a complex mechanism regulating elongation and/or cell division. This suggestion is supported by the finding that mutants with morphological anomalies present differences in their phospholipid composition (STARKA & MORAVOVÁ, 1970; MICHEL *et al.*, 1975 & 1977). Further support can be found in the report of VANDERWINKEL *et al.* (1981) stating that peptidoglycan hydrolases are sensitive *in vitro* to changes in phospholipidic environment, and also in the above mentioned report of PIZARRO *et al.* (1985) demonstrating that growth of the autolytic strain in the presence of ethanol induced the correction of abnormally high diphosphatidylglycerol concentration, finally resulting in the biochemical and phenotypical normalization of the *envD* mutant DA361.

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