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CELL DIVISION IN A SPECIES OF ERWINIA

XIII. LEAKAGE OF PROTEINS FROM THE PERIPHERAL AREA OF FILAMENTOUS CELLS

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SUMMARY

Cells of Erwinia sp. made filamentous by growth in the presence of D-serine are deficient in cell wall mucopeptide and leak proteins into the medium. Leakage is defined as release of proteins from the outside peripheral area of the cell as opposed to movement of proteins from the cytoplasm through a damaged cell membrane. Three compounds (pantoyl lactone, spermine and Carbowax-400) which can prevent or reverse division inhibition induced by D-serine, do not cause repair of mucopeptide synthesis, but do allow retention of the peripheral area proteins; most particularly pantoyl lactone. Maximum release of proteins appears to occur after filamentous growth is initiated. Attempts to stimulate division activity by adding released proteins to non-dividing cells were unsuccessful. Also, addition of antibodies, prepared against released proteins, does not inhibit division of cells growing in the absence of division inhibiting compounds.

INTRODUCTION

We have reported[1] that "leakage" of protein and nucleic acid occurs from non-dividing cells of Erwinia sp. and such cells also possess decreased amounts of cell-wall mucopeptide. As a result of these findings, it was suggested that inhibition of cell division occurs because of "secondary damage" to the cell membrane. Our suggestion seemed logical primarily because hypertonic conditions and, in particular, pantoyl lactone significantly reduce leakage and trigger division activity without repair of mucopeptide synthesis. Additional studies[2] tend to reinforce our belief that the cell membrane occupies a central role in cell division since this structure appears to initiate and sustain the invagination (pinching) type mechanism of cell division found in this organism. We have also reported[3] that DNA synthesis is not inhibited by any compound which we have studied that causes inhibition of cell division although inhibition of division of the nuclear body is very evident in filamentous cells[4,5].

The leakage phenomenon was not rigorously defined primarily because it was assumed to be the result of a "damaged" membrane best described as "porous" or one possessing larger than desired "holes". Also, cultures of non-dividing cells always contain filamentous ghost forms which indicates lysis has occurred; such an obser-

vation tends to minimize possible significance of extracellular protein and nucleic acid.

Continued investigation has shown that the mechanism(s) involved in leakage from non-dividing cells is more subtle than first imagined particularly since it has been observed that washed filamentous cells possess a reduced capacity for uptake of aspartic acid[3]. Because of this observation and our success in devising conditions wherein filamentous cells can be grown that exhibit only minimal amounts of lysis, it has been both necessary and possible for us to investigate the leakage phenomenon in greater detail.

MATERIALS AND METHODS

Cells of Erwinia sp. were grown in the following basal medium (per 100 ml): 250 mg dextrose; 480 mg NH_4Cl; 3 mg $MgSO_4 \cdot 7\ H_2O$; 174 mg K_2HPO_4; 136 mg KH_2PO_4 and trace mineral salts[6]. If D-serine ($2.1 \cdot 10^{-2}$ M) or DL-serine ($4.2 \cdot 10^{-2}$ M) is added to this medium at time of inoculation or during logarithmic growth, only minimal inhibition of cell division occurs (see Table I). However, if either β-alanine or pantothenic acid ($5 \cdot 10^{-5}$ M) is also added, profound inhibition of cell division results[3] (see also Table I). To obtain relatively large amounts of filamentous cells, we added β-alanine at time of inoculation and either D- or DL-serine 12–13 h later after relatively good growth had occurred. Incubation was then continued, usually for 6 additional hours for maximal cell elongation. Other possible additives made at 12–13 h included: pantoyl lactone $7.7 \cdot 10^{-2}$ M; spermine $6.0 \cdot 10^{-3}$ M; or Carbowax-400 4%.

Concentration of released proteins was accomplished by dialysis after first sedimenting the cells and filtering the spent medium through a 0.45-μm Millipore filter. Dialysis was done at 2–4° in 2 changes (each 24 h) of buffer ($1.44 \cdot 10^{-1}$ M Tris, pH 8.0) wherein the inside–outside volume ratio was 1–20 and finally against distilled water containing 15% Carbowax-6000 (usually to 1–2 ml which takes about 12–18 h).

For release of surface proteins from control cells, the cold-shock procedure of HEPPEL[7] was utilized. When necessary, these proteins were concentrated by dialysis directly against 15% Carbowax-6000 or by lyophilization.

Polyacrylamide disc gel electrophoresis (detergent system for resolution of envelope components) was accomplished as previously described[8]. The non-detergent gel system was the same as described in the Buchler manual for operation of the Polyanalyst unit using anionic gel systems (pH 9.3), but without stacking gel.

To prepare antibodies to proteins released during growth or by cold shocking, rabbits were inoculated both intramuscularly and subcutaneously using a total volume of 0.9 ml (4 mg protein in 50% glycerol) once each week for three consecutive weeks. Antibodies were precipitated from collected sera using $(NH_4)_2SO_4$ according to WEIR[9].

Antibody titers were obtained on whole serum using the precipitin procedure; these titers usually ran from 1–80 to 1–160 (4 + reactions). Final concentration of purified γ-globulin employed in growth experiments was approx. 24 mg/ml of growth medium.

Adenosine triphosphatase activity was estimated in the following way: To 5.1 ml of buffer–salts solution (0.016 M Tris–HCl, pH 8.0 which was 8 mM in KCl and 0.01 M in $MgCl_2$) was added 0.1 ml of cell suspension washed two times in Tricine buffer (0.01 M, pH 6.8) and 0.8 ml ATP solution (4.3 mM dipotassium ATP made up fresh in the buffer–salts solution). Incubation was on a Dubnoff metabolic shaker for

60 min at 37°. Amount of inorganic phosphate released was measured using a molybdo-vanadate method[10].

RESULTS

Culture conditions devised for this study have as their basis the combined ability of β-alanine (or pantothenate) and D-serine to inhibit division of cells growing in a glucose–high ammonia medium without appreciable lysis[3]. Growth curves for each nutritional circumstance are given in Fig. 1. Relationships between cell size, growth response and amount of protein released into the medium under the various conditions are shown in Table I.

Maximum release of proteins occurs when cells are grown in the presence of both serine and β-alanine (situation 4); however, release is caused primarily by serine (compare situations 2 and 3). Although three compounds (pantoyl lactone, Carbowax-400 and spermine) promote division activity, a positive correlation to amount of protein released is not always evident. It is very clear however that release of proteins is greatly suppressed by pantoyl lactone.

Judging from the distances traveled in gel columns (Fig. 2), it can be seen that, regardless of the growth situation wherein release occurs, there is little or no qualitative difference in the proteins released; rather, differences are quantitative.

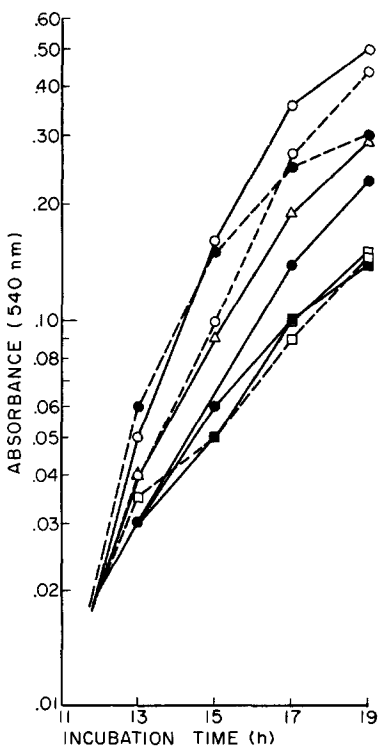


Fig. 1. Growth of cells after 12 h in the presence and absence of different additives. ○—○, no additions; ○---○, β-alanine; ●---●, DL-serine; ●—●, DL-serine and β-alanine; △—△, pantoyl lactone; ■—■, DL-serine, β-alanine and pantoyl lactone; □—□, DL-serine, β-alanine and spermine; □---□, DL-serine, β-alanine and Carbowax-400.

TABLE I

EFFECT OF DIFFERENT ADDITIVES ON GROWTH, CELL SIZE AND PROTEIN RELEASE

All additions to the basal medium were made after 13 h growth except β-alanine which was added prior to inoculation.

Growth situation	*Absorbance at 540 nm (19 h)*	*Average cell size (μm)**	*Protein released (% of cell dry wt.)*
(1) Basal medium	0.50	3.5 (2–4.5)	0.0
(2) + β-Alanine	0.44	2.5 (2–4)	0.0
(3) + DL-Serine	0.30	6.0 (3–11)	0.5
(4) + β-Alanine and DL-serine	0.23	28.0 (7–40)	1.7
(5) + Pantoyl lactone	0.29	2.0 (1.5–2.5)	0.0
(6) Situation No. 4 + pantoyl lactone	0.14	3.0 (2–4.5)	0.0
(7) Situation No. 4 + Carbowax-400	0.14	6.5 (4.5–9.5)	0.9
(8) Situation No. 4 + spermine	0.15	4.0 (2.5–6.5)	1.5

* Heat-fixed preparations stained with crystal violet; number given is the average of about 50 direct measurements. The number given in parentheses indicates the range in size observed.

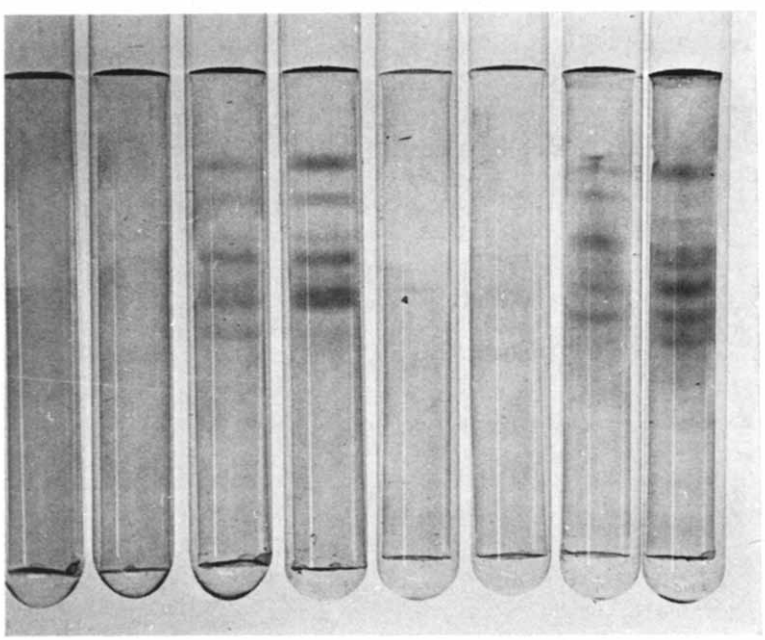
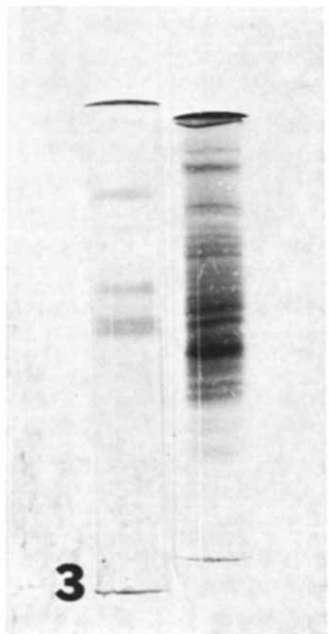


Fig. 2. Polyacrylamide gel electrophoretic columns (non-detergent system) of proteins released into the medium by cells growing in the presence and absence of different additives. Gels appear in the same sequence as given in Table I. Volumes of material electrophoresed were adjusted to dry weight of the culture in order to permit some quantitative assessment of proteins present.

To determine if released proteins are the result of cell lysis (cytoplasmic origin), cells were broken by passage through an X-press and the supernatants analysed. Comparison of major and minor band positions as well as number of bands resolved (Fig. 3), reveals that released proteins are not the result of general cell lysis.

To determine if released proteins come from the periplasmic space, control cells



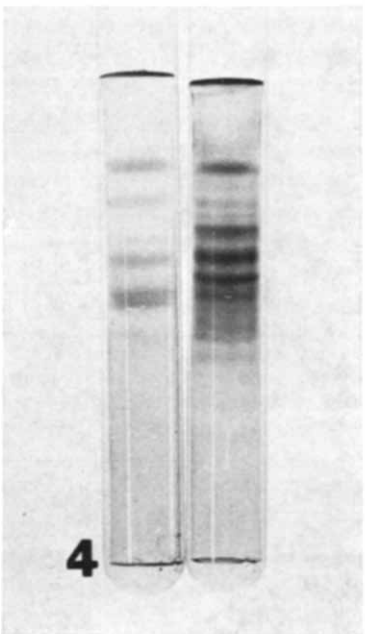


Fig. 3. Polyacrylamide gel electrophoretic columns (non-detergent system) of proteins released into the medium by growing filamentous cells (left side) and proteins present in the cytoplasm of normally growing cells (right side).

Fig. 4. Polyacrylamide gel electrophoretic columns (non-detergent system) of proteins released into the medium by growing filamentous cells (left side) and proteins released from normal cells by the cold osmotic shock procedure (right side).

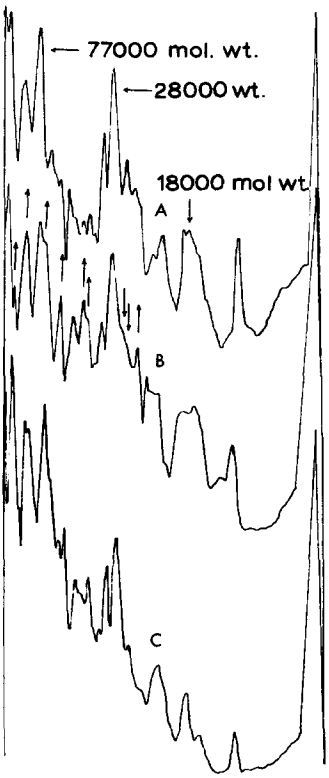
were cold-shocked utilizing the procedure of HEPPEL[7] (filamentous cells could not be studied in this way since they undergo extensive lysis during shocking). The representative gel given in Fig. 4 shows that all bands of released proteins from filaments migrate to the same positions as some of the proteins obtained by cold osmotic shocking. Judging from the number of bands resolved, cold shocking causes the release of more proteins. Because of the number present as well as their migratory behavior on gel columns, the conclusion appears warrented that proteins released from filamentous cells during growth originate, at least in part, from the periplasmic space. This is particularly true if it is accepted that all proteins released by the cold-shock procedure originate from the periplasmic space area. Because fewer proteins are released from filamentous cells during growth than can be released from control cells by the cold-shock procedure and because we cannot rule out a cell wall origin for some of the released proteins[11], it appears advisable to define the release area as the cell periphery (cell wall *plus* periplasmic space) rather than limiting it to the periplasmic space area as defined by HEPPEL.

To determine if some of the released proteins are leached from the envelope of filamentous cells, normal and filamentous cells were broken by passage through an X-press and washed envelopes analyzed. Gel scans (Fig. 5) reveal that the envelope of filamentous cells contains decreased amounts of two proteins (arrows pointing down); however, it is also clear that the concentration of several proteins primarily in the higher

molecular weight area is increased (arrows pointing up). Significantly, the pattern is more nearly normal in pantoyl lactone-grown cells. Increases in certain membrane proteins have also been observed in non-dividing cells of *Micrococcus lysodeikticus* disIIp$^+$ as well as a "normalizing" of them in the presence of pantoyl lactone[12].

Hydrolysis of adenosine triphosphate (ATPase) is an enzyme activity intimately associated with the cell membrane of bacteria[13–15]. Therefore, presence or absence of this enzyme activity should also give some indication whether or not damage to the surface of the cell membrane occurs in filamentous cells. In preliminary studies, we observed that whole cells of Erwinia sp. can be used to quantitatively assay for presence of this activity. The experimental findings shown in Fig. 6 demonstrate two points. (1) Amount of ATPase activity is inversely proportional to age of culture in control cells. (2) Filamentous cells in early culture time contain about 40% less ATPase activity than control cells. As these filamentous cells continue to grow and become progressively smaller in average size, ATPase activity increases before beginning what appears to be a normal downward trend during later culture time.

ATP cannot penetrate the cell membrane of organisms; therefore, presence of ATPase activity in whole cells indicates that the enzyme activity is extra-cytoplasmic.



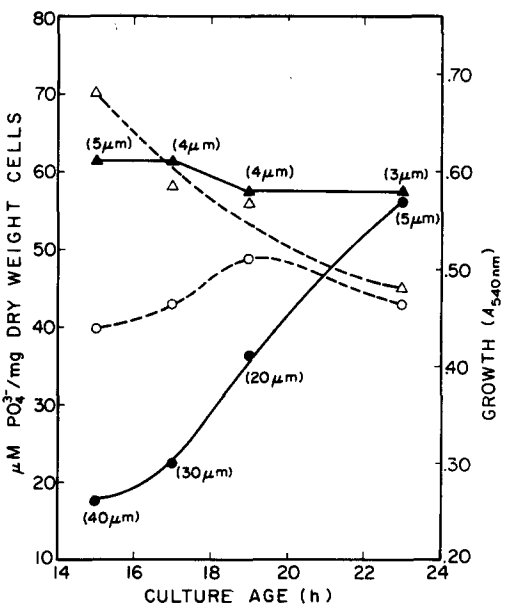


Fig. 5. Scans of gel columns (detergent system) of envelope proteins from control cells (A), filamentous cells grown in the presence of β-alanine and serine (B) and cells grown in the presence of β-alanine, serine and pantoyl lactone (C).

Fig. 6. Growth and average cell size of normal (▲—▲) and filamentous cell forms (●—●) and ATPase activity of normal (△---△) and filamentous cells (○---○) at various culture ages.

Further evidence which indicates localization of the activity in the peripheral area of these cells can be summarized as follows. (1) Cold shocking causes about a 45% drop in normal cell enzyme activity content. (2) Trypsinization of normal cells (30 min at 25° in bicarbonate buffer, pH 8.4) causes about a 20% reduction in activity. (3) Based on the findings of PARDEE AND WATANABE[16], treatment of cells with diazonaphthalene disulfonate will inactivate surface-located proteins. We have observed that treatment of normal cells of Erwinia sp. for 20 min at 25° with this compound eliminates all ATPase activity.

It has been somewhat unusual to note that either sonication or X-pressing releases about 80% of the ATPase activity in a soluble form (non-sedimentable after centrifugation at 105000 × *g* for 2 h) which tends to indicate a cytoplasmic origin. It should be pointed out however that if a water-soluble protein is held in the periplasmic space by a cell wall through which it cannot normally pass, such protein will be present with the cytoplasmic fraction in any broken cell preparation.

HEPPEL[7] reported that proteins released by cold shock are not essential for cell division; however, he also observed that replenishment of these proteins occurs at a relatively rapid rate when the cells are placed back into a favorable growth medium. Release of proteins appears to be occurring continually during growth of filaments; therefore, such cells are different and might be best described as growing in a state of chronic osmotic shock.

To determine if the time and rate of protein release could be correlated to induction of division inhibition, growth media were analyzed for released proteins during the time that cells were becoming filamentous. As shown in Fig. 7 greatest release of protein occurs after cells exhibit the largest increase in size. It is also shown, however, that as cells begin to shorten, less release of protein occurs. Overall, these

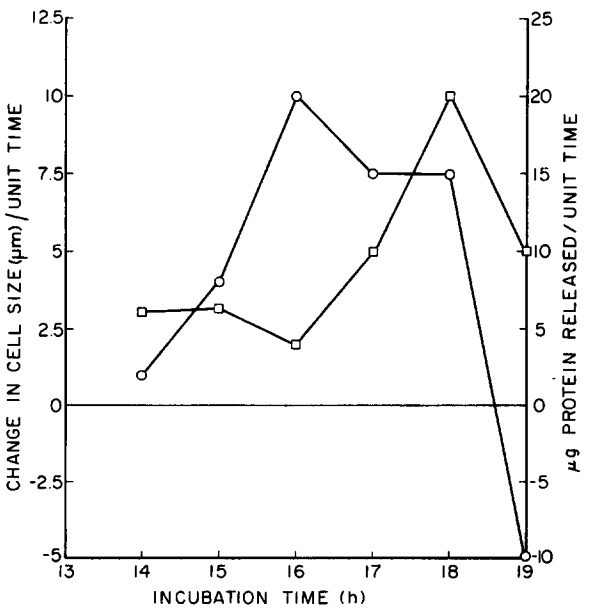


Fig. 7. ○—○ indicates change in cell size per unit time; □—□ shows the amount of protein released on a time basis.

TABLE II

MUCOPEPTIDE COMPONENTS IN THE ENVELOPE OF CELLS GROWN UNDER DIFFERENT CONDITIONS

Mucopeptide components analyzed utilizing the procedure of GUIRE[17].

Growth situation and concn. of additives	*μM/mg envelope dry wt.**			*% Decrease****
	Diamino-pimelic acid	*Glucosamine***	*Muramic acid*	
Glucose–NH_4Cl	0.0438 (1)	0.1496 (3.4)	0.0408 (0.93)	—
+ D-Serine ($2.09 \cdot 10^{-2}$ M) and β-alanine ($5 \cdot 10^{-5}$ M)	0.0324 (1)	0.1277 (3.9)	0.0310 (0.96)	25
+ D-Serine, β-alanine and pantoyl lactone ($7.68 \cdot 10^{-2}$ M)	0.0257 (1)	0.1218 (4.7)	0.0288 (1.1)	35
+ D-Serine, β-alanine and spermine ($5.9 \cdot 10^{-3}$ M)	0.0284 (1)	0.0919 (3.2)	0.0303 (1.1)	30
+ D-Serine, β-alanine and Carbowax-400 (4%)	0.0272 (1)	0.0702 (2.6)	0.029 (1.1)	35
Glucose–aspartic acid	0.0475 (1)	0.1291 (2.7)	0.0514 (1.1)	—
+ Ultraviolet light	0.0277 (1)	0.0969 (3.5)	0.0389 (1.4)	33

* Molar ratios given in parentheses with diaminopimelic acid set at 1.

** Ratio of glucosamine to diaminopimelic and muramic acid in pure mucopeptide is 1:1:1 (GRULA *et al.*[2]). Extra glucosamine is present in lipopolysaccharide (GRULA AND ABEGAZ, unpublished).

*** Calculated using only those figures for diaminopimelic and muramic acid.

data indicate that the lesion(s) leading to inhibition of division is also involved in the release of proteins.

Since release of proteins could be due in part to decrease in cell wall mucopeptide, various cell forms of Erwinia sp. were analyzed using a column method that permits quantitation of diaminopimelic acid[17]. Pertinent data given in Table II confirm and extend our previous findings[1] and show that less mucopeptide is present in the cell wall of all cells grown in the presence of β-alanine and D-serine regardless of whether or not inhibition of division is prevented by pantoyl lactone, Carbowax-400 or spermine.

Varying concentrations, either of proteins released from growing filamentous cells or these obtained by cold-shocking normal cells, were added to ultraviolet-irradiated cells[18] at time of inoculation or after varying periods of growth (up to 12 h) to determine if such preparations could cause prevention or reversal of division inhibition. Only negative data were obtained.

When our experiments were initiated, we were aware that some binding proteins released by cold shocking will at times apparently re-attach themselves and function whereas, at other times, they will not[19,20].

Others[21] have reported doing experiments similar to ours and obtaining positive results (division was stimulated); however their protein-containing material was released by normally growing cells of *Escherichia coli*.

On the basis of gel migration, we have determined that ultraviolet-produced filaments also release proteins similar to those shown in Fig. 2. Therefore, such fila-

mentous cells should have suitable attachment sites available. As additional information, data presented in Table I confirm[1] that treatment with ultraviolet light causes a significant decrease in cell wall mucopeptide.

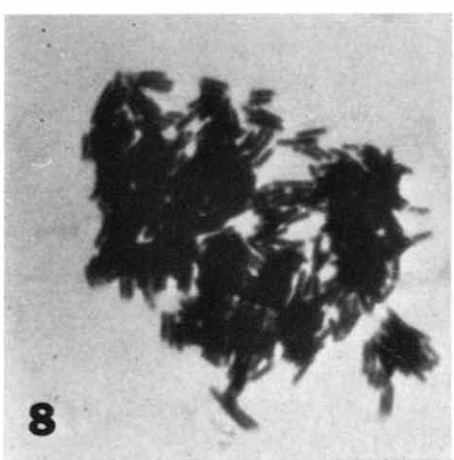
Antibodies were also prepared to proteins released from filaments to see if they would inhibit cell division. Theoretically, the antibodies should combine with the proteins *in situ* hopefully inhibiting the cell division mechanism if the proteins are required for cell division. As controls, antibodies were also prepared against proteins released from normal cells by the cold-shock procedure.

Regardless of the time of antibody addition to logarithmically growing normal cells, inhibition of division was never observed. Unfortunately, again, there is no way of knowing for certain if the antibodies reach their correct destination. Antibodies to material released from filamentous cells do react at the surface of normally growing cells since agglutination occurs quickly (Fig. 8). Agglutination is very poor however when antibodies to proteins released from control cells by cold-shocking are used. Antibodies were also added to D-serine-induced spheroplasts (GRULA AND ABEGAZ, unpublished procedure) which agglutinate quickly in the presence of antibodies to material released from filamentous cells, but not to antibodies prepared against proteins released by cold-shocking (Fig. 9).

To help antibody molecules to pass through the wall area, cells were pre-treated with EDTA (0.1 mM, pH 7.4, for 30 min at 25°). In no instance were treated cells inhibited from dividing when placed back into growth media either with or without antibody molecules.

Antibodies were also added to normal cells prior to irradiation, hopefully to combine with and thus help protect the peripheral proteins from possible damaging effects of ultraviolet light. No protection was evident since irradiated cells grew into long filaments after inoculation into the glucose–aspartic acid growth medium.

Agglutination was always more rapid and extensive when antibodies to released materials were added either to spheroplasts or normal cells. We suspected, therefore,



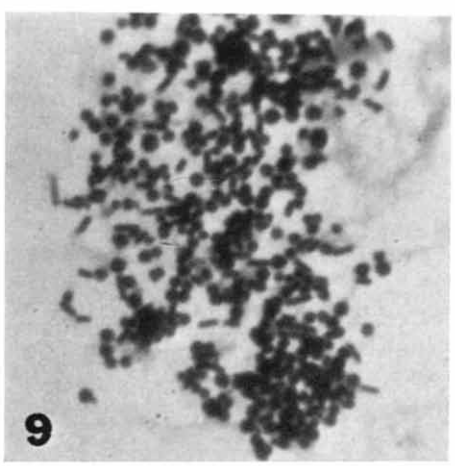


Fig. 8. Agglutinated cells of Erwinia sp. which were grown for 5 min in the presence of antibodies to material released from growing filamentous cells.

Fig. 9. Agglutinated rod and spheroplast forms of Erwinia sp. treated as in Fig. 8.

that antigenic components of the outer cell wall of filamentous cells are released along with peripheral proteins. WEINBAUM[22] reported that the envelope of branched and filamentous forms of *E. coli* B have a greatly decreased content of lipopolysaccharide, but increased amounts of protein.

To determine if lipid was present in released protein preparations, such material was dried by lyophilization, extracted using chloroform–methanol (2:1, v/v) and analyzed by thin-layer chromatography (plates contained Silica Gel G and were developed in chloroform–methanol–water (65:25:4, v/v/v)).

Few if any membrane fragments are present since phosphatidyl-ethanolamine, the most abundant phospholipid in the envelope (membrane?) of this organism was never observed. A relatively large amount of non-phospholipid-type lipid having an R_F of about 0.20 was present however.

We also tested released material for carbohydrates such as 2-keto-3-deoxyoctonate and 3,6-dideoxyhexoses which appear to be present in all cell wall lipopolysaccharides thus far examined[23]. Analysis was made by determining color produced in the presence of 2-thiobarbituric acid[24] after periodate oxidation at 55°. About 0.13% of the released components (dry wt. basis) are thiobarbituric acid positive.

Because of these observations, we are concluding that some wall lipopolysaccharide is also released from growing filamentous cells.

DISCUSSION

The major significance of the data presented is to permit a redefinition of the leakage phenomenon as it occurs in non-dividing cells of Erwinia species. Knowing that protein molecules don't come through the cell membrane of filamentous forms of this organism, but, rather, are released from the peripheral region permits us to understand how large protein molecules get into growth medium while the cell membrane is able to retain semi-permeable characteristics.

The mechanism for protein release is not understood; however, filamentous cells of Erwinia sp. are deficient in mucopeptide (Table II and ref. 1). Also, as shown in Fig. 7, rate and extent of release is greatest after filamentous growth is discernible. It seems reasonable therefore to suggest that a deficiency in mucopeptide allows for mechanical removal of loosely held peripheral proteins via a continued washing process by the growth medium. It is evident that damage to the cell wall of bacteria is not an isolated cellular event, but should, instead, be viewed in relation to other properties and functions of the cell.

Regardless of the ultimate reason(s) for release of these proteins, it is also highly significant that pantoyl lactone negates both the inhibition of cell division and release of peripheral proteins in the absence of any discernible repair to cell wall mucopeptide. Thus, the action of pantoyl lactone must somehow be directed toward the cell membrane, particularly since we have also observed (unpublished data) that presence of pantoyl lactone inhibits uptake of several compounds by normal cells. Further, (Fig. 5) presence of pantoyl lactone prevents, to a significant extent, the large quantitative changes seen in envelope proteins from filamentous cells.

Our inability to demonstrate involvement of released proteins in the division process should be accepted with reservations because of inherent difficulties in these types of experiments. It may, perhaps, be more significant that release of proteins

occurs after inhibition of division is discernible. It might also be argued that release of relatively large amounts of proteins in the presence of spermine, wherein cell division occurs, argues against a need for these proteins in division; however, it is possible that spermine somehow mimics the action of one or more of the released proteins.

Overall, we feel it is significant that three different types of changes have now been demonstrated in the periphery of filamentous cell forms of Erwinia species. These are: (1) decreased content of cell wall components such as mucopeptide; (2) release of several peripheral proteins with presumed enzyme activity such as the demonstrated ATPase activity; (3) decrease in at least two detergent-soluble envelope proteins with concomitant increases in several others. Which change occurs as the primary event and which bears a direct relationship to cell division activity remain yet to be established. Apparently the changes that accrue even to the proteins within the envelope complex of these filamentous cells are more extensive than those reported for temperature-sensitive filamentous mutants of *E. coli*[25,26].

ACKNOWLEDGEMENTS

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