JOURNAL OF BACTERIOLOGY, May 1974, p. 334-341
Vol. 118, No. 2

Printed in U.S.A.

Electron Microscope and Autoradiographic Study of Ultrastructural Aspects of Competence and Deoxyribonucleic Acid Absorption in *Bacillus subtilis*: Ultrastructure of Competent and Noncompetent Cells and Cellular Changes During Development of Competence

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Received for publication 21 November 1973

By means of electron microscope autoradiography of component cultures of *Bacillus subtilis* exposed to [^{3}H]thymidine-labeled transforming deoxyribonucleic acid competent and noncompetent cells can be distinguished. Competence is not limited to a specific phase of the cell division cycle. With serial section electron microscopy of competent and noncompetent cells, two types of mesosomal structures are observed: mesosomes connected to the plasma membrane only (plasma membrane mesosomes) and mesosomes which are additionally connected to the nuclear bodies (nuclear mesosomes). The two types show different cellular distributions. Especially the number of nuclear mesosomes is higher in competent than in noncompetent cells. This, and the observation that the increase and decrease of competence is correlated with both the number of cells carrying nuclear mesosomes and the number of nuclear mesosomes per cell, suggests that mesosomes are involved in the acquisition of competence.

Various properties differentiate competent from noncompetent cells: (i) increased resistance to the bactericidal action of penicillin (17), (ii) reduced cellular length (8, 13), (iii) reduced deoxyribonucleic acid (DNA) (2, 15) and ribonucleic acid (RNA) synthesis (15), (iv) decreased density, enabling separation of competent and noncompetent cells by density gradient centrifugation (4, 12), and (v) chromosomes with replication points arrested in the vicinity of the replicative origin (6). With regard to the number of nucleoids, it has been reported that competent cells tend to be uninucleate (3, 23). However, Singh and Pitale's (23) light microscopy analysis of competent cells obtained by gradient centrifugation does not exclude the possibility that binuclear cells may enter the competent state. By using a competence regimen different from that used by Singh and Pitale (23), Erickson and Copeland (8) observed, after density gradient centrifugation of competent *Bacillus subtilis* cultures, that the fraction enriched for competent cells contained mainly binuclear cells.

Data obtained from electron microscopy of thin sections of *B. subtilis* cells suggest an increase in the total volume of mesosomes during the phase of maximal competence (30). Because the actual increase in competence was not measured in that study, the results did not provide information as to what extent the increase was correlated with the development of competence.

The present study was undertaken to obtain more detailed information with respect to the possible involvement of mesosomes in the development of competence in *B. subtilis* by using single and serial section electron microscopy of competent and noncompetent cells of cultures having developed and developing competence.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* 168 strain 2G-12 (*ade trpC2*), described previously (25), was used as recipient. Transforming DNA was obtained from strain OG-1 (wild type) and strain 2G-8 (*thy tyr*$_1$).

Preparation of DNA. Transforming DNA was prepared as described earlier (25). [^{3}H]thymidine-labeled DNA was isolated from 2G-8 cells growing in the presence of 3 μg of [^{3}H]thymidine (5-methyl-T, Radiochemical Centre, Amersham, England) per ml, with a specific activity of 19.6 Ci/mmol.

Competence regimen. As described previously (25), *ade trpC2* cells were grown to maximal competence either by method A (casein hydrolysate supple-

mented medium) or by method B, in which casein hydrolysate was replaced by the competence-enhancing amino acids advised by Wilson and Bott (28).

Transformation procedure. For electron microscopy-autoradiography *ade trpC2* cells grown to maximal competence by method B were used. Samples of the recipient culture were incubated for 7.5 min at 32 C either with wild-type DNA (3 μg/ml) or with [^{3}H]thymidine-labeled *thy tyr*$_1$ DNA (1.6 μg/ml, specific activity 1.7×10^7 dpm/μg) in the presence of nonradioactive thymidine (120 μg/ml) and gently aerated. DNA uptake was terminated by further incubation for 1 min with bovine pancreatic deoxyribonuclease (DNase, 125 μg/ml; British Drug Houses) in the presence of 0.03 M $MgSO_4$. Cells exposed to wild-type DNA were plated on appropriately supplemented agar plates for the determination of the transformability of the cultures. Samples (20 ml) exposed to [^{3}H]thymidine-labeled DNA were chilled in crushed, frozen medium (20 ml), washed on membrane filters (Millipore Corp., HAWP 04700) with 80 ml of fresh medium at 0 C, and resuspended in 20 ml of fresh medium. Subsequently, samples (3 ml) were diluted with fresh medium (3 ml at 0 C), centrifuged, and resuspended in ice-cold fresh medium (3 ml).

In the experiment designed to study the ultrastructural morphology of the cells during competence development, cells growing to competence according to method A were used. At 30-min intervals during growth of the culture at 37 C and after the dilution step, cells were withdrawn from the culture, centrifuged at room temperature, and used for electron microscopy. Simultaneously, cells were exposed to wild-type DNA (3 μg/ml) for 30 min at 37 C, treated with DNase (20 μg/ml) for 5 min, and plated on appropriately supplemented agar plates to determine the increase in competence.

Electron microscopy. Cells were fixed in OsO_4, treated with uranyl acetate, dehydrated in ethanol, and embedded in methacrylate-divinylbenzene (14) as described previously (30). Thin sections (approximately 50 nm) were cut on an LKB Ultratome I microtome equipped with a diamond knife and transferred to copper grids (200 or 300 mesh/inch; Veco, The Netherlands) covered with a carbon-coated Formvar film. Serial sections were mounted on special grids (R 22 A; Veco). Sections were examined in a Philips EM300 electron microscope at 60 or 80 kV with a 25-μm objective aperture. Micrographs were taken with a 35-mm camera on Kodak fine-grain positive film, developed in Kodak HBP developer (1:6), and printed on Rapido print paper (Agfa-Gevaert).

Electron microscopy-autoradiography. Sections on grids were coated with a 5-nm thick carbon layer by evaporation, mounted on alcohol-cleaned objective slides, and overlaid with a monolayer of Ilford L.4 Nuclear Research emulsion by using the wire loop technique (5). The emulsion was prepared as described previously (22).

Autoradiographs were stored for 6 or 25 weeks at 4 C in light-tight boxes containing silica gel and were developed according to the method of Wisse and Tates (29), which involves gold latensification and elon ascorbic acid development. This method was modified in such a way that the preparations were developed for 4 min instead of 7.5 min. After fixation with hardener fixer (3 min), the grids were carefully removed from the objective slides, dipped into distilled water, and dried at room temperature.

RESULTS

Recognition of competent cells by electron microscopy-autoradiography. In order to recognize the competent cells of a culture by autoradiography, it is necessary that two conditions are fulfilled. Firstly, the individual competent cells should be given sufficient opportunity to absorb radioactive DNA and, secondly, the exposure time of the autoradiographs should be sufficiently long, so that the photographic emulsion over a labeled cell will carry silver grains.

We have shown previously (25) that an incubation period of 5 min at 37 C with saturating amounts of DNA is sufficient to enable all competent cells of a culture to absorb DNA from the medium. If the culture is exposed at 32 C for 7.5 min, approximately the same transformation frequencies are observed as compared with incubation at 37 C for 5 min, indicating that under the former conditions, used in the present case, all competent cells were able to incorporate DNA.

Autoradiographs of serial sections were developed after 25 weeks of exposure and used for comparing competent with noncompetent cells. The total number of grains per labeled cell appeared to be 20.9 ± 17.4. Because the large majority of the competent cells is uniform with respect to the capacity to absorb DNA (25), the high average number of grains observed per labeled cell indicates that all cells which absorbed [^{3}H]thymidine-labeled DNA will appear as labeled cells in autoradiographs.

Phase of cell division. In order to determine whether competence of individual cells is related to a specific phase of the division cycle, competent and noncompetent cells were classified in one of the following categories: (i) no septum present, (ii) an incomplete septum present, and (iii) a complete septum present. The results obtained from serially sectioned cells (Table 1) show that the distribution of the various divisional stages in the competent fraction is not significantly different from that in the noncompetent fraction ($\chi^2 = 2.70$, $0.30 > P > 0.20$). The data suggest that the competent state is not associated with a particular phase of the cell's division cycle. The serial sections were also used to determine the mean number of

grains per labeled cell in the three classes (figures between parentheses in Table 1). The data show that the phase of the division cycle is not correlated with the amount of DNA absorbed. This conclusion is in accordance with the data presented previously (25).

Number of nucleoids in competent and noncompetent cells. By examining predominantly the sectioned cells from which the results presented in the previous paragraph were obtained, the number of nucleoids per cell was determined. DNA-containing areas not visibly separated were considered to represent a single nucleoid. Table 2 shows that approximately 50% of the competent cells have one nucleoid, whereas the remainder, consisting of approximately 90% of cells with a partial or complete septum, are binuclear. The ratio of both cell types in the noncompetent fraction is slightly, but not significantly, different ($\chi^2 = 2.22$, $0.20 > P > 0.10$).

Mesosomal types. The advantage of serial section over single-section electron microscopy is the possibility of tracing the mesosomes. Mesosomes are invariably connected to the plasma membrane. However, with respect to their being connected to other cell structures, two types can be distinguished: (i) those which are connected to the plasma membrane only, and (ii) those which are additionally connected to the nucleoids. The first type will be designated as plasma membrane mesosomes, the second as nuclear mesosomes.

Number of mesosomes in competent and noncompetent cells. With the aid of serial-section electron microscopy of cells exposed to radioactive donor DNA, the number of both types of mesosomes was determined in competent and noncompetent cells. As is shown in Table 3, competent cells have an increased number of nuclear mesosomes as compared with noncompetent cells ($t = 4.86$, $P < 0.001$ for cells without a septum; $t = 3.94$, $P < 0.001$ for cells with a partial or complete septum). To a lesser extent, this also applies to plasma membrane mesosomes ($t = 2.08$, $0.05 > P > 0.02$ for cells without a septum; $t = 4.68$, $P < 0.001$ for cells with a partial or complete septum). The results also suggest that cells having a septum have a higher probability of carrying mesosomes than cells without a septum.

Position of mesosomes. The intracellular position of both types of mesosomes was determined as follows. The length of cells without a septum was measured on photographs of serial sections in order to locate the middle of each cell. The distance from the tip to the middle was then divided into three equal parts, so that in each cell half a tip (T), a tip-middle intermediate (TMI), and a middle (M) zone were distinguished. In cells with a septum, the distance from the tip to the septum was measured and both cell halves were divided into five zones: a tip (T), a tip-middle intermediate (TMI), a middle (M), a middle-septum intermediate (MSI) and a septum (S) zone. Figure 1 presents the zonal division made. It should be noted that the M zones in cells without a septum will pass into future S zones and that the M zones in cells with a septum will pass into M zones of future daughter cells. The distribution of the mesosomes over the various cellular zones was obtained by determining, in serially sectioned cells, the zone in which the mesosomes are connected with the plasma membrane.

The distribution (in percentage) of the total number of each type of mesosome over the various zones of both cells with and without a septum is presented in Fig. 2, which shows that neither competent nor noncompetent cells contain an appreciable number of nuclear mesosomes in T zones. With the exception of noncompetent cells with a septum, the nuclear mesosomes are most frequently observed in M zones. The distribution of nuclear mesosomes in competent cells lacking a septum is almost identical to that in noncompetent cells ($\chi^2 = 0.02$, $P > 0.99$). However, with respect to their distribution in cells having a septum, significant differences exist between competent and noncompetent cells ($\chi^2 = 23.66$, $P < 0.01$).

The distribution of plasma membrane mesosomes is very much different from that of the

TABLE 1. *Distribution of various divisional stages in competent and noncompetent cells*

Cells	No septum	Incomplete septum	Complete septum	Total no. of cells examined
Competent	49.4 (22.8)[a]	28.6 (19.1)[a]	22.0 (17.0)[a]	91
Noncompetent	47.6	38.1	14.3	84

[a] Average number of silver grains per cell.

TABLE 2. *Distribution of cells with one and two nucleoids in competent and noncompetent cells*

Cells	No. of nucleoids		Total no. of cells examined
	1	2	
Competent (%)	51.5	48.5	97
Noncompetent (%)	62.5	37.5	88

TABLE 3. *Average number of each of the two mesosomal types in competent and noncompetent cells*

Cells	Septum status	Avg no. of nuclear mesosomes	Avg no. of plasma membrane mesosomes	Total no. of cells examined
Competent	Without a septum	1.08 ± 0.86	3.20 ± 2.77	25
	Having a septum	1.73 ± 1.14	4.57 ± 3.04	30
Noncompetent	Without a septum	0.29 ± 0.46	2.15 ± 1.31	41
	Having a septum	0.86 ± 0.74	2.16 ± 1.57	43

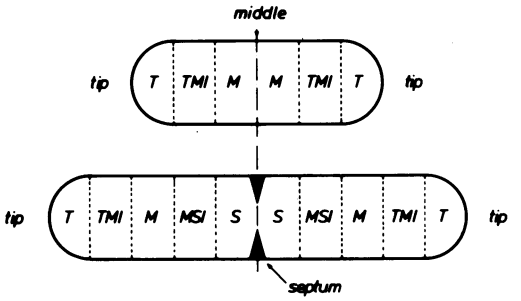

FIG. 1. *Schematic representation of the zonal partition of cells with and without a septum (see text). T, Tip; TMI, tip-middle intermediate; M, middle; S, septum; MSI, middle-septum intermediate.*

other mesosomal type. Most frequently they occupy the T zones of cells without a septum and T and S zones of cells with a septum, whereas their frequency in M zones is relatively low. The distribution of this type of mesosome over the various zones in competent cells without a septum is statistically equal to that in noncompetent cells (χ^2 = 2.29, 050 > P > 0.30). This also applies to cells with a septum (χ^2 = 6.05, 020 > P > 0.10). From these results it is concluded that the two types of morphologically distinguishable mesosomes are differently distributed over the cell and that competent cells are distinguishable from noncompetent cells with respect to the distribution of nuclear mesosomes, with the restriction that the differences are manifest only when the cells are in a particular phase of the division cycle.

Cellular alterations during development of competence. An *ade trpC2* culture grown by method A for 2.5 h at 37 C in Spizizen minimal medium supplemented with glucose (0.5%), casein hydrolysate (0.02%), and the appropriate growth requirements (20 μg/ml) was diluted twofold with fresh medium without casein hydrolysate and the growth requirements and was incubated for 2.5 h at 37 C. At 30-min intervals after the dilution step, samples were taken for determining the size of the competent fraction with Goodgal and Herriott's (11) method and

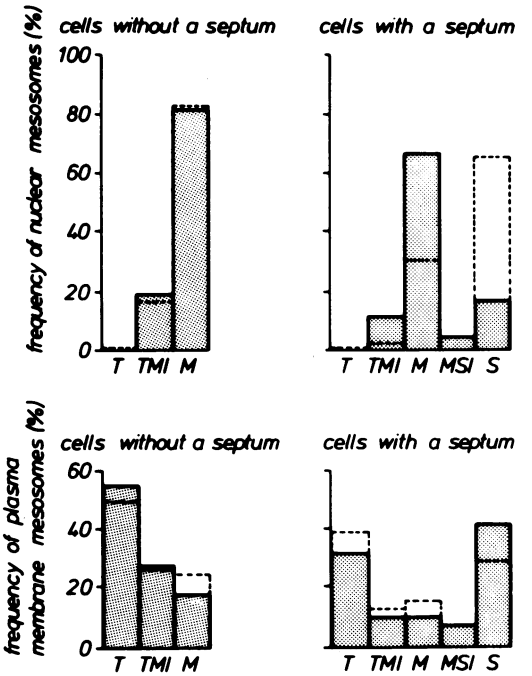

FIG. 2. *Intracellular distribution of mesosomes in competent and noncompetent cells. The site of attachment of the mesosomes to the plasma membrane was determined in 55 labeled cells (25 without a septum) and 84 nonlabeled cells (41 without a septum). Solid lines represent the distribution in competent cells, and interrupted lines that in noncompetent cells. See Fig. 1 for abbreviations.*

for studying intracellular changes on the electron microscopic level. To that purpose, both single sections (1,000 per sample) and serial sections (30 cells, 10 to 15 sections per cell) were used.* The results are presented in Fig. 3 and show that the competent fraction increases from 5 to 25% during the first 2.5 h, remains constant for approximately 30 min, and starts to decrease gradually afterwards (Fig. 3a). The percentage of cell profiles with a nuclear mesosome increases by a factor of 3.5 to 4 during the first 2.5 h and after the next 30 min decreases also (Fig. 3b).

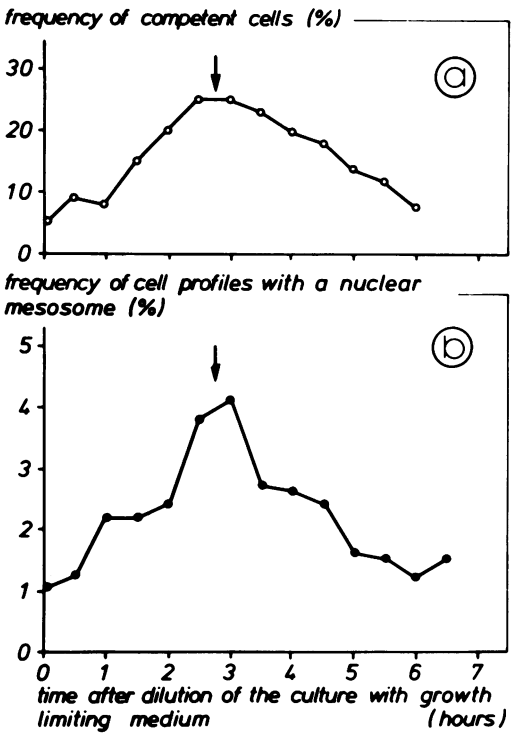

FIG. 3. *Percentage of cell profiles with nuclear mesosomes during increase in competence. With 30-min intervals after dilution of an ade trpC2 culture with growth-limiting medium, samples were incubated for 30 min at 37 C with wild-type DNA (3 μg/ml), treated with DNase (20 μg/ml) for 5 min, and plated in order to determine the size of the component fraction by Goodgal and Herriott's (11) method (a). From each sample, cells were fixed for electron microscopy examination, and 1,000 random cell profiles were used to determine the percentage of cell profiles with nuclear mesosomes (b). The arrow indicates the phase of maximal competence. The percentages of transformation in the phase of maximal competence were: ade$^+$, 1.47; trpC2$^+$, 1.03; and ade$^+$ trpC2$^+$, 5.9 × 10^{-2}.*

Because the two types of mesosomes are not distinguishable in single sections, serial sections of a number of cells from each sample were used to estimate the variation in the frequency of cells having (i) nuclear mesosomes, (ii) exclusively plasma membrane mesosomes, and (iii) for estimating the variation in the average number of both types of mesosomes per cell during increase of competence. The frequency of cells with nuclear mesosomes, which almost without exception also carry one or more plasma membrane mesosomes, increases from 50 to 95% during the first 3.5 h and then gradually decreases (Fig. 4a). Also, the average number of nuclear mesosomes per cell increases by a factor of 3 during the first 3 h and then decreases (Fig. 4b). The fraction of cells having exclusively plasma membrane mesosomes decreases during development of competence and increases again during the gradual loss of competence (Fig. 4a), whereas the average number of mesosomes of this type decreases during the first 2 h, then increases for 2.5 to 3 h and, finally, at the end of the experimental period, decreases again (Fig. 4b). These results show that a fairly good correlation exists between increase and decrease of competence and the change in both the frequency of cells with nuclear mesosomes and the average number of such mesosomes per cell.

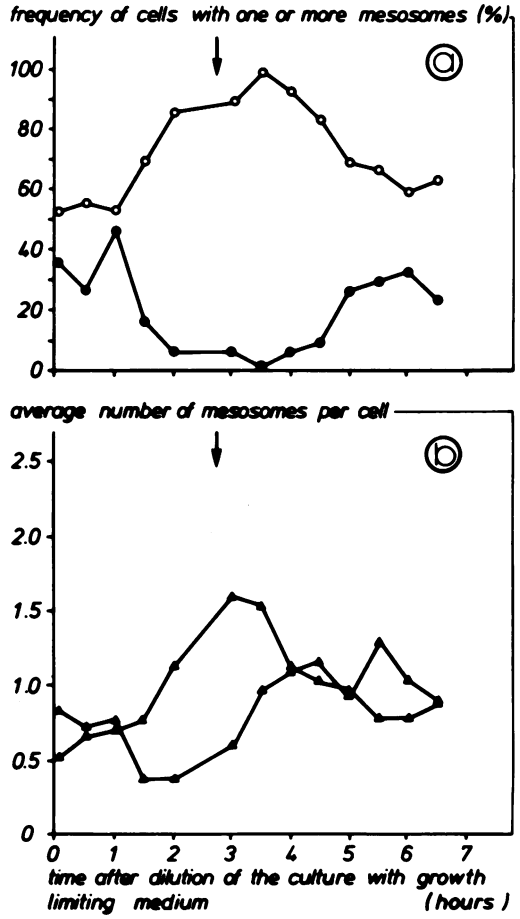

FIG. 4. *Fraction of cells with mesosomes and the average number of mesosomes per cell during increase of competence. (a) ○, Frequency of cells with nuclear mesosomes; ●, frequency of cells which have exclusively plasma membrane mesosomes. (b) △, Average number per cell of nuclear mesosomes; ▲, average number per cell of plasma membrane mesosomes. The arrow indicates the phase of maximal competence.*

However, the correlation is by no means numerically complete; during the phase of maximal competence approximately 25% of the cells are competent (Fig. 3a), whereas 90% of the cells carry a nuclear mesosome, implying that the mere presence of the connecting mesosomal type is insufficient to render cells competent.

The data presented in this paragraph, showing that the pattern of development and loss of competence is correlated with variation in both the number of nuclear mesosomes and the frequency of cells having this mesosomal type, agree with the autoradiographic data showing that, as compared with noncompetent cells, competent cells have an increased number of mesosomes, especially of the connecting type.

DISCUSSION

The autoradiographic analysis shows that approximately 50% of the competent cells contain two nucleoids. This result disagrees with indirect evidence in favor of uninuclearity of competent cells (3, 23). Because 45% of the binuclear competent cells have a completed septum, these cells may be considered as uninuclear cells which have not yet separated. Singh and Pitale's (23) results do not exclude the possibility of a competent state in binuclear cells. Their results, obtained by sucrose gradient centrifugation of a competent *B. subtilis* population after incubation with DNA, suggest a correlation between the distributions of uninuclear cells and transformants in the gradient. Because the maximally competent fractions contain about 50% binuclear cells, the existence of binuclear competent cells was by no means excluded. Our results show that competence is not restricted to uninuclear cells; in that sense they confirm data reported by Erickson and Copeland (8). In an X-ray study of competence development in *B. subtilis*, Epstein (7) suggested that the precompetent population consists of a mixture of uninucleate and binucleate cells; the binucleate cells should become mononucleate in a medium provoking competence. It is clear that our results do not support the possibility that the development of the competent state requires the conversion of binuclear to mononuclear cells.

Considerable differences exist between competent and noncompetent cells with respect to the average number of mesosomes, especially the nuclear mesosomes and their distribution over the various cell zones in cells developing or having developed a septum. The observations that competent cells have more nuclear mesosomes than noncompetent cells and that increase of competence is correlated both with the increase in the frequency of cells with these mesosomes and their average number per cell suggest that the nuclear mesosomes are involved in the development of competence. However, noncompetent cells also carry nuclear mesosomes. In addition, the increase in the average number of nuclear mesosomes per cell during increase of competence cannot exclusively be attributed to an increase of these connections in future competent cells, because in that case a high concentration of the connecting mesosomal structure should be observed in a relatively small fraction of cells. This is not the case; the maximal number observed per cell never exceeds three. Also, no quantitative correlation exists between the increase in the fraction of cells having nuclear mesosomes and the increase in the competent fraction. Therefore, it seems likely that all cells, but especially the future competent cells, contribute to the increase in the average number of nuclear mesosomes per cell during development of competence.

There is little or no correlation between development of competence and either the average number per cell of plasma membrane mesosomes or the frequency of cells carrying this type of mesosome exclusively. Nevertheless, this type of mesosome is more frequently observed in competent than in noncompetent cells. Therefore, on the basis of the present analysis, plasma membrane mesosomes cannot be excluded from being involved in competence development. If they are precursors of nuclear mesosomes, their exclusion from being involved in development of competence would seem unwarranted. It is conceivable that transition between the two mesosomal types exists, because the increase in the average number per cell of nuclear mesosomes during increase of competence, though not completely, is paralleled by a decrease in that of plasma membrane mesosomes. Conversely, the decrease in number of nuclear mesosomes during decrease in competence is accompanied by an increase in the number of plasma membrane mesosomes (Fig. 4b).

We are ignorant concerning the way mesosomes might be involved in the cell's development of the ability to incorporate high-molecular-weight DNA from the medium. Because, with the possible exception of mesosomes being essential for the synthesis of penicillinase in *Bacillus licheniformis* (20), there is no evidence for a specific enzymatic function of mesosomes (9, 10, 18, 19, 21, 24), it is difficult to imagine

that a specific enzyme, or set of enzymes, is associated with the mesosomes which creates the cell's ability to incorporate DNA. However, it is conceivable that the local increase in membrane surface results in a local increase in the concentration of membrane-bound enzymes. Apart from the function of binding and of transport of transforming DNA of mesosomes (27), we might envisage that the increase in membrane surface results in high concentrations of autolytic enzymes in the surroundings of the mesosomal site of attachment to the plasma membrane. A relationship between competence and autolytic action has often been suggested (1, 31, 32).

The finding that the two mesosomal types, initially distinguished on the basis of their being connected to the nucleoid, are differently distributed lends support to the supposition that they are different. If they can interconvert, their different distribution indicates that this occurs in specific regions of the cell.

ACKNOWLEDGMENTS

The authors thank W. J. Feenstra for a critical discussion and M. Veenhuis for excellent technical assistance.

The present investigations have been carried out with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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