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Autolytic Activity Associated with Competent Group H Streptococci

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Competent cells of group H streptococci strains Wicky and Challis autolyzed markedly when placed at 37 C in 0.05 M tris(hydroxymethyl)methyl-amino-propane sulfonic acid buffer (*p*H 9.0 to 9.1) containing 0.02 M 2-mercaptoethanol, whereas noncompetent cells autolyzed slightly. Autolysis of competent Wicky cells did not occur at 0 C or after the cells were heated at 100 C for 5 min. Culture fluids derived from strain Challis that contained competence factor (CF) activity did not contain lytic activity. Addition of native deoxyribonucleic acid (DNA) to competent Wicky cells caused a retardation in the rate of autolysis; ribonucleic acid and alkali-denatured DNA had less of an effect. Supernatant fluids derived from competent cell lysates lysed noncompetent Wicky cells but were inactive against cells of *Hydrogenomonas eutropha*, a group A *Streptococcus*, and against a commercial lysozyme substrate (*Micrococcus lysodeikticus*). This lytic activity was inactivated by heat (5 min at 100 C). Electron microscopic observations of autolyzed cells showed that autolysis occurs only at the site of cross-wall formation. A close relationship between the development of competence and autolysis is suggested by the fact that certain conditions that prevent the establishment of the competent state in Wicky populations (such as no CF, addition of CF simultaneously with chloramphenicol, and addition of trypsin-inactivated CF) also prevent autolysis. This observation emphasizes the indirect or inductive nature of CF on these processes.

The ability of strains of some bacterial species to take up and integrate deoxyribonucleic acid (DNA) molecules is a poorly understood phenomenon called competence. A role therein for autolytic enzymes was previously implied (1, 30). Young and Spizizen (29) demonstrated a lytic enzyme in cell walls derived from competent and noncompetent cells of *Bacillus subtilis*. Although the enzyme (an *N*-acylmuramyl-L-alanine amidase; 30) was more active in cell walls derived from competent cells, the extent of cell wall degradation was the same in cell walls derived from noncompetent cells. It was speculated that this enzyme makes holes in the cell wall and thereby enables the penetration of DNA into the cells (1, 30). This hypothesis has been strengthened by the finding of Akrigg and Ayad (2). These authors showed that a column fraction containing competence factor activity for *B. subtilis* cells also contained a lytic enzyme. However, in both of the experiments reported above, the lytic activity was greatest when the cells reached the end of exponential growth and not necessarily when they were maximally competent.

This report presents more direct evidence that the development of competence in group H streptococcal cells is mediated by an autolytic enzyme(s). It was fortuitously found that only competent cultures, of either strains Wicky or Challis, autolyze when placed in buffer containing a reducing agent, at *p*H values greater than 7.0. Noncompetent cells do not autolyze (or autolyze slightly) under the same conditions.

MATERIALS AND METHODS

Organisms. The organisms used in this report were group H streptococci strains Wicky and Challis. The Wicky strain was an erythromycin-resistant mutant (resistant to 1.5 μg of erythromycin per ml) isolated in this laboratory: it produces no competence factor (CF) and is incompetent in the absence of added CF. The Challis strain was one that produces CF and develops competence in defined media (12).

Growth and maintenance of organisms. Wicky cells were grown and maintained in a medium (BHI-HS) consisting of Brain Heart Infusion (Difco) supplemented with 2.5% (v/v) heat-inactivated (56 to 60 C for 30 min) horse serum (Microbiological Associates). Challis cells were grown and maintained in the synthetic growth medium, MS 6 (11, 12). Stationary cultures of both strains were stored in 0.5-ml amounts at −70 C in their respective media.

Competence development. (i) Thawed Wicky cells (0.5 ml) were diluted with BHI-HS (5 or 12 ml) and incu-

bated statically overnight at 37 C. Five milliliters of the overnight culture was added to 100 ml of BHI-HS (warmed to 37 C) contained in a 250-ml cotton-stoppered Erlenmeyer flask and incubated for 40 min. A saturating amount of CF was then added for 50 min. At this time period, Wicky cells are fully competent. Noncompetent Wicky cells were obtained by incubating similarly treated cells for 50 min in the absence of CF. (ii) Challis cells were made competent in the following way. A thawed culture (0.5 ml) was diluted to 12 ml with MS 6 and incubated statically 18 to 24 hr at 37 C. Five milliliters of this culture was then added to 100 ml of BHI-HS (warmed to 37 C) contained in a cotton-stoppered 250-ml Erlenmeyer flask. The cells were incubated 2 hr at 37 C, at which time they were fully competent. Challis cultures incubated only 40 min in BHI-HS are not competent; Challis cultures incubated 180 min are less competent than 2-hr cultures.

Transformation. When transformation was determined, 0.8 ml of "competent cells" was added to 0.1 ml of a chloramphenicol (CAP) solution (100 μg/ml) and 0.1 ml of a DNA solution (80 μg/ml), and incubated for 30 min at 37 C. [CAP prevents additional competence from developing, but it does not prevent cells that are already competent from taking up DNA (18, 20).] At the end of this period, the cells were diluted appropriately in 0.4% (w/v) BHI and plated with Brain Heart agar (BHA). The plates containing transformed cells were incubated for 3 hr at 37 C, at which time they were overlaid with an equal volume of BHA containing dihydrostreptomycin (600 μg/ml). Plates containing appropriate dilutions for the determination of the number of viable colonies in each sample were not overlaid. Colonies were counted after 40 hr of incubation at 37 C. The transformation frequency (per cent) equals the number of transformed colonies per milliliter $\times$ 100 divided by the number of viable colonies per milliliter.

Transforming DNA. Transforming DNA was isolated from cells of a group H *Streptococcus* strain SBE: 12, that is resistant to more than 1 mg of dihydrostreptomycin per ml. The method of Marmur was used (14), except that cell lysis was obtained with the muralysin derived from the infection of the group C streptococcal strain 26 RP66 with the C-1 bacteriophage (3, 9, 15).

Alkali denaturation of DNA. Transforming DNA and calf thymus DNA (Sigma; both at 1 mg/ml in 0.85% NaCl) were titrated to *p*H 13 with 2 N NaOH. After 10 min at 25 C, the DNA solutions were brought to *p*H 7.4 with 0.5 M NaH_2PO_4.

CF. CF was obtained from culture supernatant fluids of strain Challis and used without further purification. Different preparations were used and included: (i) CF made in a chemically defined medium (CF-S; 12), (ii) CF made in a chemically defined medium containing 1% (w/v) Neopeptone (17; Difco) and heat-inactivated horse serum (CF-NS), and (iii) CF made in BHI-HS (CF-BS). CF-BS and CF-NS were produced by strain Challis after 3 hr of incubation at 37 C. All CF preparations were filtered for sterilization (0.22-μm pore size; 7103 filter, BioQuest) and titered before use. All CF preparations were stored at −40 C.

Lysing buffer. The lysing buffer consisted of 0.05 M tris(hydroxymethyl)methyl-amino-propane sulfonic acid (TAPS), containing 0.01 to 0.02 M 2-mercaptoethanol (2-ME), at *p*H 9.0 to 9.1.

Treatment of cells. Wicky or Challis cells were grown as described above, with or without CF, and harvested by centrifugation (4,500 rev/min, SS-1 rotor, Sorvall centrifuge, 0 to 5 C, 20 min). The cells were washed once with 5 ml of cold 0.85% (w/v) NaCl and suspended in 2.5 ml of cold deionized water.

Autolysis. The cells were diluted 1:10 into cold lysing buffer and incubated at 37 C. Optical density (OD) values were determined at 550 nm with a Beckman model B spectrophotometer. All reported values are based on the starting OD taken as the 100% value. The starting OD ranged from 0.350 to 0.480.

Electron microscopy. Samples of control cultures in BHI-HS or MS 6, and competent and noncompetent cells incubated in lysing buffer for various time periods, were negatively stained with 2% (w/v) phosphotungstic acid (*p*H 7.2) directly on carbon-Formvar-coated copper grids. For sectioning, similar samples were fixed in equal volumes of 5% (w/v) cacodylate-buffered glutaraldehyde (*p*H 7.2; reference 23) for 1 hr at room temperature. Secondary fixation in acetate-Veronal-buffered 1% (w/v) osmium tetroxide at room temperature, and subsequent procedures, followed the methods of Ryter and Kellenberger (22), except that embedment was made in Epon 812 (13) from propylene oxide. Sections, silver-gray to gold, were cut with a diamond knife on an LKB Ultratome I and gathered on Formvar-coated copper grids. All sections were stained for 3 min with alkaline lead citrate (21). Examination and photography, at plate magnifications from 6,000 to 50,000, were done with an Hitachi electron microscope (model HU-11C) operated at 75 kv.

RESULTS

The initial observation of competent cell autolysis was made when the cells were incubated in 0.025 M tris(hydroxymethyl)aminomethane (Tris) buffer containing 0.025 M maleic acid (Tris-maleate), 5×10^{-3} M 2-ME, and 5×10^{-3} M ethylenediaminetetraacetic acid (at *p*H 6.5 to 7.0). However, the observed rate of autolysis was slow. Subsequent experiments showed that at *p*H values of 8.8 to 9.1 the autolysis rate was rapid. (*p*H values greater than 9.1 were not tested.) After these initial experiments, the Tris-maleate buffer above was replaced by the TAPS buffer containing only 0.02 M 2-ME. A rapid rate of autolysis was also observed in 0.02 M ammonium carbonate containing 0.02 M 2-ME (*p*H 8.6) and in 10^{-5} M NaOH containing 0.02 M 2-ME (*p*H 9.0). Competent cells do not autolyze in 0.025 M Tris-maleate buffer containing 0.02 M 2-ME at *p*H values below 6.5, in 0.05 M TAPS buffer containing 0.02 M 2-ME (*p*H 4.8), in deionized water (*p*H 5 to 6), or in 0.02 M 2-ME (*p*H 5 to 6).

Autolysis of competent and noncompetent Wicky cells. Figure 1 shows the kinetics of autolysis of competent and noncompetent Wicky cells diluted in TAPS buffer containing 2-ME (*p*H 9.0

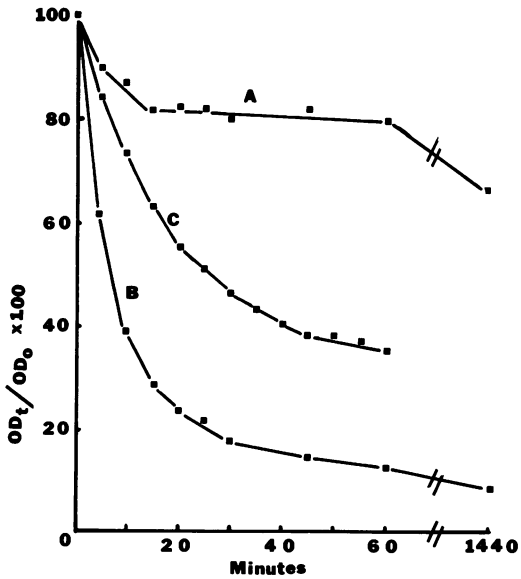


FIG. 1. *Autolysis of competent and nonccompetent Wicky cells. Wicky cells were grown and treated for autolysis. A, Noncompetent Wicky cells in lysing buffer; B, competent Wicky cells in lysing buffer; C, competent Wicky cells in TAPS buffer (pH 9.0 to 9.1) without 2-ME.*

to 9.1). In about 7 min, 50% of the competent culture (transformation frequency, 7.5%) underwent lysis (curve B); after overnight incubation, the final OD was 9% of the starting OD. Figure 1, curve A shows the degree of lysis in noncompetent cultures (transformation frequency, 8.6×10^{-5}%). The maximum change in OD observed with these cells was 34% after overnight incubation. Figure 1, curve C shows the rate of autolysis of competent Wicky cultures that were suspended in only TAPS buffer (*p*H 9.0 to 9.1). It is apparent that autolysis is enhanced by the reducing agent. Dithiothreitol could effectively replace 2-ME.

Autolysis of competent and noncompetent Challis cells. Figure 2 shows the autolysis rate of competent and noncompetent Challis cells. In this experiment, Challis cells were harvested at 40, 120, and 180 min of incubation in BHI-HS. Forty-minute cells were not competent (transformation frequency, 1.3×10^{-4}%) and decreased in OD by about 42% after overnight incubation in TAPS buffer containing 2-ME, at 37 C (curve A). Two-hour cells were the most competent (transformation frequency, 1.9%) and lysed to 50% of the starting OD in about 45 min (curve B). Three-hour cells were of lower competence (transformation frequency, 0.05%) and lysed to 50% of the starting OD in about 60 min (curve C).

Lysis of noncompetent Wicky cells. Figure 3 shows the rate of lysis of noncompetent Wicky cells when they were added to a crude autolysate of competent Wicky cells (curve A). (Similar results were obtained with a cell-free autolysate, centrifuged at 3,000 $\times$ *g* for 30 min at 2 C.) These data imply that the autolysin (s) is free in solution and that noncompetent Wicky cells contain its substrate. Competent Wicky cells also lysed when added to an autolysate. Similar experiments done with *Hydrogenomonas eutropha* cells, cells of a group A *Streptococcus*, or *Micrococcus lysodeikticus* cells were negative (no lysis).

Is CF a lysin? Since noncompetent Wicky cells do not autolyze spontaneously in lysing buffer, these cells were used to test whether CF per se had lytic activity. CF did not cause noncompetent Wicky cells to lyse and, therefore, is not itself a lysin. Moreover, CF did not inhibit autolysis when added to competent Wicky cells in lysing buffer.

Effect of chloramphenicol on the development of competence and autolysis. CAP inhibits the induction of competence development when added simultaneously with CF to Wicky cells (20). It was of interest to determine whether CAP (10 μg/ml) also inhibited the induction (or synthesis) of the autolysin in Wicky cells when added with

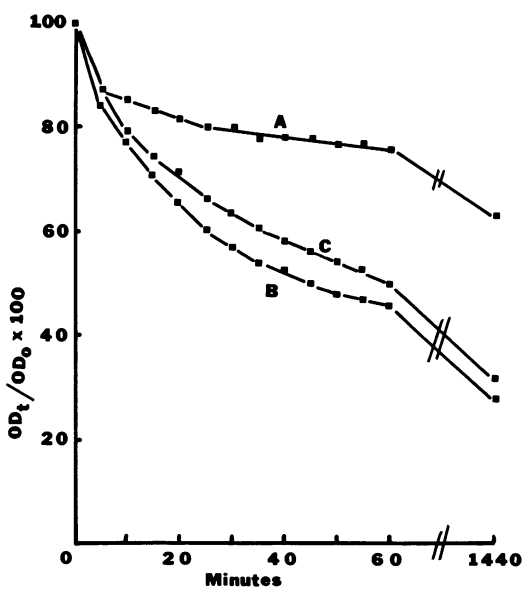


FIG. 2. *Autolysis of competent and noncompetent Challis cells. Challis cells were grown and treated for autolysis. See text for their respective levels of competence as judged by their frequencies of transformation to dihydrostreptomycin resistance. A, Forty-minute Challis cells; B, 120-min Challis cells; C, 180-min Challis cells.*

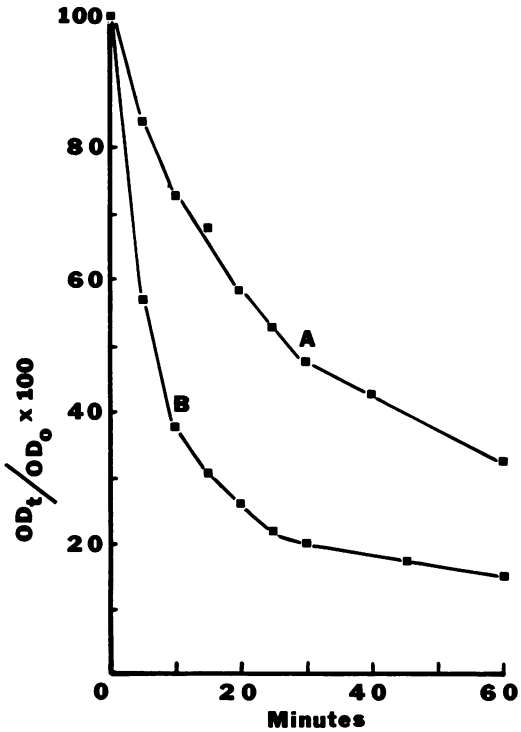


FIG. 3. *Lysis of noncompetent Wicky cells by a crude competent Wicky cell autolysate. Competent and noncompetent Wicky cells were prepared. The competent cells were allowed to autolyse for 60 min at 37 C (curve B), at which time noncompetent Wicky cells were added directly to the crude autolysate (curve A).*

CF. Figure 4 shows that this is indeed the case. The extent of autolysis observed was similar to that obtained with noncompetent Wicky cells (curve A). The transformation frequencies obtained in this experiment were: competent cells, 6.7%; cells treated simultaneously with CAP and CF, 1.1 $\times$ 10^{-4}%. There was no reduction in cell viability with the CAP concentration used. CAP added to competent Wicky cells in lysing buffer had no effect on the autolysis rate. Therefore, the autolysin is preformed or preactivated in competent cells, as a result of a protein synthesizing step possibly induced by CF.

Effect of trypsinized CF on the development of competence and autolysis. CF-BS was inactivated with trypsin (5 μg/ml, 60 min at 37 C; Worthington Biochemical Co.) and heated at 56 C for 35 min to destroy the trypsin activity. Wicky cells treated with the trypsinized CF did not develop competence (transformation frequency, 1.8 $\times$ 10^{-4}%) nor did they autolyze (15% reduction in OD at 60 min; Fig. 5, curve A). Control cells treated with nontrypsinized but heated CF-BS developed high competence (transformation frequency, 6.1%) and autolyzed at a fast rate (curve B; 50% decrease in OD in 9 min). These data show that destruction of CF activity also destroys the cells subsequent ability to autolyse.

Effect of temperature on autolysis. Figure 6 shows that autolysis of competent Wicky or Challis cells is sensitive to temperature. Autolysis is inhibited if the cells are heated in lysing buffer at 100 C for 5 min (curves A and B) or if they are maintained in lysing buffer at 0 C (curve C). (Autolysis of competent Wicky cells was observed at 40 C.) High heat (100 C, 5 min) also destroyed the lytic activity present in cell-free autolysates derived from competent Wicky cells (Fig. 7). However, it is not yet clear whether high heat (100 C, 5 min) also affects the substrate. Addition of a cell-free autolysate to boiled competent or noncompetent Wicky cells did not cause a marked change in OD (measured over a 2-hr period), and electron microscopic observation of such treated cells did not show the morphological changes in cell walls seen in autolysate-treated

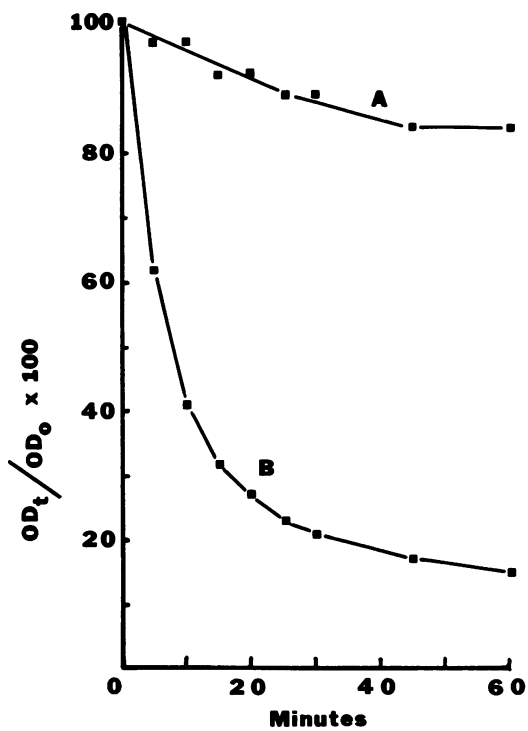


FIG. 4. *Autolysis of Wicky cells treated simultaneously with competence-factor and chloramphenicol. Two cultures of Wicky cells were grown. Competence-factor was added to one culture, and competence-factor and chloramphenicol (10 μg/ml) were added to the other. The cells were incubated 50 min at 37 C and treated as already described. A, Autolysis of Wicky cells treated with competence-factor and chloramphenicol; B, autolysis of Wicky cells treated only with competence-factor.*

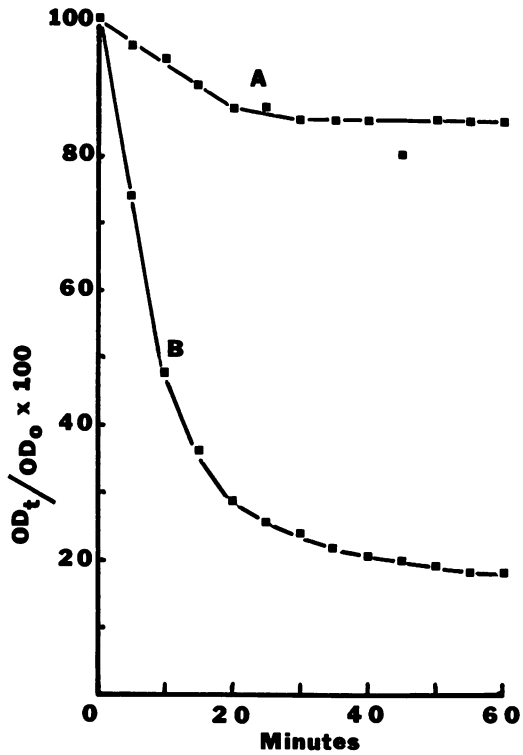


FIG. 5. *Autolysis of Wicky cells treated with trypsinized competence-factor. Two cultures of Wicky cells were grown. One culture received competence-factor that was previously treated with trypsin (5 μg/ml for 60 min at 37 C) and heated (56 C for 35 min) to destroy the trypsin activity (A). The other culture received competence-factor that was incubated for 60 min at 37 C in the absence of trypsin and heated at 56 C for 35 min (B).*

noncompetent-unheated control cells (*data not shown*). Possibly, the coagulated cytoplasm prevents the cell wall hemispheres from coming apart (Fig. 9C, E, and G) and also prevents any drop in OD, regardless of whether undetected gaps in cell wall may be present.

Since a soluble factor (s) is present in competent Wicky cell autolysates that can lyse noncompetent Wicky cells and since it is heat labile, the factor (s) is most likely an enzyme.

Physical change and site of autolytic activity. Figure 8 shows the increase in the number of lysed competent Wicky cells as a function of time in lysing buffer at 37 C. A similar picture was obtained with competent Challis cells. The changes coincide with decreasing OD values as shown in Fig. 1, curve B. Under the same conditions, noncompetent cells never exceed minimal lysis comparable to that shown in Fig. 8B.

Figure 9 compares, by both negative stain and sections, intact cells with lysed (competent) cells. The intact cells, as shown in Fig. 9A and B, have the same appearance whether they are Challis or Wicky cells in culture media, noncompetent Challis cells in lysing buffer, noncompetent Wicky cells (no CF previously added, or both CF and CAP previously added) in lysing buffer, or competent cells in lysing buffer at 0 C. The lysed cells (Fig. 9C–G) are representative of the majority of cells of a competent culture (either Challis, or Wicky plus CF) after a maximum decrease in OD values was obtained in lysing buffer at 37 C. Appropriate sections reveal minimal breaks in the cell-wall to be always at the equatorial site of cross-wall formation, which thus appears to be the site of autolytic activity (arrows, Fig. 9D). Extrusion of membrane-bound protoplasmic blebs (Fig. 9C, F, G) or of strands of DNA (Fig. 9D, F) through such gaps is common, and occasionally what appear to be mesosomal filaments or vesicles are seen extruding or extruded (Fig. 9F, arrow). However, much of the cytoplasmic membrane, some polysomes, and occasional membranous configurations remain within the cell wall. These features can also be seen in negatively stained preparations (Fig. 9C), in which,

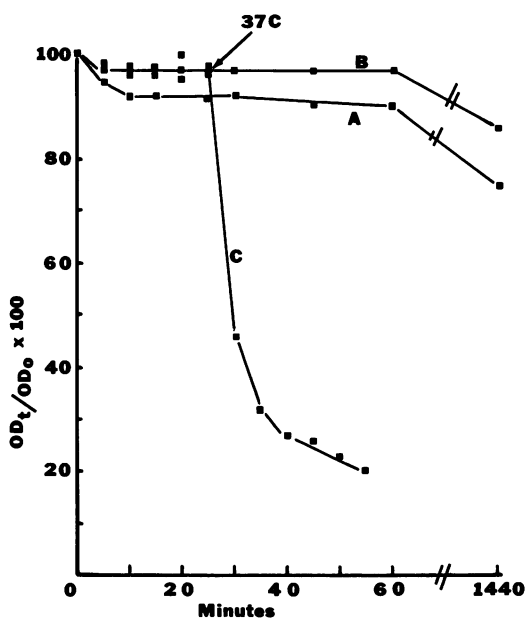


FIG. 6. *Effect of temperature on the autolytic rate in competent Wicky and Challis cells. Wicky and Challis cells were made competent. Samples from each culture were added to boiling lysing buffer and heated at 100 C for 5 min. They were then cooled to 37 C, and turbidities were read at the intervals shown. A, Wicky cells; B, Challis cells. Another sample of Wicky cells was added to lysing buffer that was chilled to 0 to 4 C. These cells were allowed to incubate at 0 to 4 C for 25 min, at which time they were transferred to 37 C (C).*

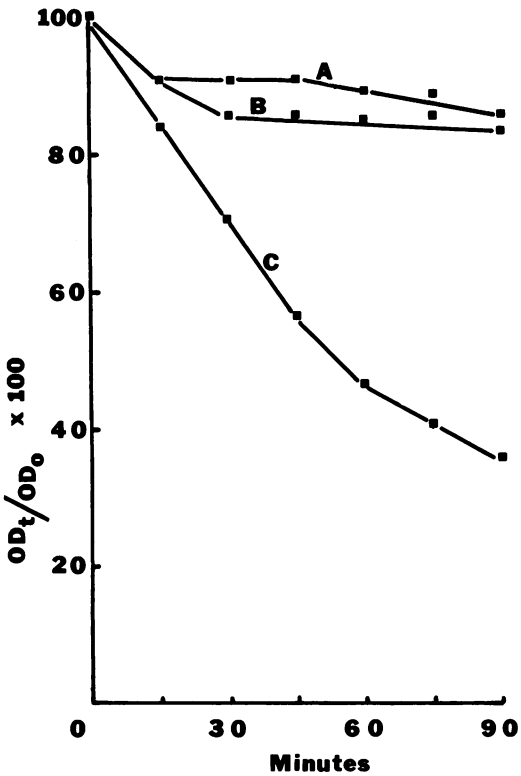


FIG. 7. *Heat inactivation of the lytic factor derived from competent Wicky cell autolysates. Wicky cells (400 ml of culture) made competent as described in the text were allowed to autolyze for 2 hr at 37 C in 4 ml of lysing buffer. The lysate was clarified by centrifugation (30 min at 2,000 × g, 4 C), and the clear supernatant fluid was used as the source for the lytic factor. Noncompetent Wicky cells were prepared as described. A, Noncompetent Wicky cells in lysing buffer containing 0.4 ml of heated (100 C, 5 min) lytic factor; B, noncompetent Wicky cells in lysing buffer with no addition; C, noncompetent Wicky cells in lysing buffer containing 0.4 ml of nonheated lytic factor. Incubation temperature was 37 C.*

probably because of forces resulting from drying and flattening, the small equatorial gaps are exaggerated (Fig. 9C, E, G) or an occasional cell wall hemisphere is completely separated and lost (Fig. 9E). Even after 24 hr of incubation at 37 C in lysing buffer, the appearance is unchanged from that shown. There is no ultrastructural evidence of further cell wall degradation nor of degradation at sites other than the cross-wall, and the common picture is one of cell wall hemispheres held in approximation by remnants of cytoplasmic membrane.

Effect of DNA and RNA on the rate of autolysis. If the autolysin that is demonstrable only in competent cells plays a role in cellular competence, then one might expect that the addition of DNA to such cells may somehow influence autolytic activity. When competent Wicky cells were first treated with transforming DNA for 30 min before harvesting for lysis, the initial rate of autolysis was reduced. Fifty per cent lysis of competent Wicky cells not treated with DNA was obtained in 6 min (Fig. 10, curve B), whereas 50% lysis of competent Wicky cells treated with DNA occurred in 23 min (Fig. 10, curve A). At the time of testing, the DNA was resistant to the action of deoxyribonuclease. DNA added to competent Wicky cells in lysing buffer did not influence the autolysis rate.

The observed retarding effect by DNA on the rate of autolysis was not limited to transforming DNA. Calf thymus DNA (8 μg/ml) produced the same effect (Fig. 11, curve A). This retardation in lytic activity could be reduced by using alkali-denatured calf thymus DNA (Fig. 11, curve B) or alkali-denatured transforming DNA (Fig. 10, curve C). Ribonucleic acid (RNA, 8 μg/ml; Torula yeast; Sigma), added to competent Wicky cells as above, had no effect.

Table 1 shows that competent Wicky cells treated with native calf thymus DNA at a concentration that retarded the rate of autolysis also reduced the frequency of transformation (70% inhibition). On the other hand, the same concentration of alkali-denatured calf thymus DNA retarded the rate of autolysis to a lesser extent and also reduced the frequency of transformation to a lesser extent (52% inhibition). Alkali-denatured transforming DNA that lost 99% of its biological activity did not retard the rate of autolysis or affect the frequency of transformation.

Effect of autolysis on cell viability and transformation. The loss of cell viability followed over a 2-hr period essentially paralleled the fall of OD units. At 2 hr, when approximately 80% of the culture lysed, the viable cell count also decreased by 80%. With earlier time periods, the correlation was not exact.

Although the OD decreased slightly in noncompetent cultures (Fig. 1, curve A), there was no detectable decrease in cell viability. If one cell in a chain of cells lysed, a viable colony would still result.

Cells from a competent Wicky population that remained viable after 60 min of autolysis were isolated and tested again for competence and autolysis. Both events occurred with the same frequencies and rates as the original culture. Resistant cells, therefore, are not genetic variants.

Experiments similar to the one above done with competent Wicky cells treated with transforming DNA before lysis showed that when 50% of the initial OD was left (84% of the cells still

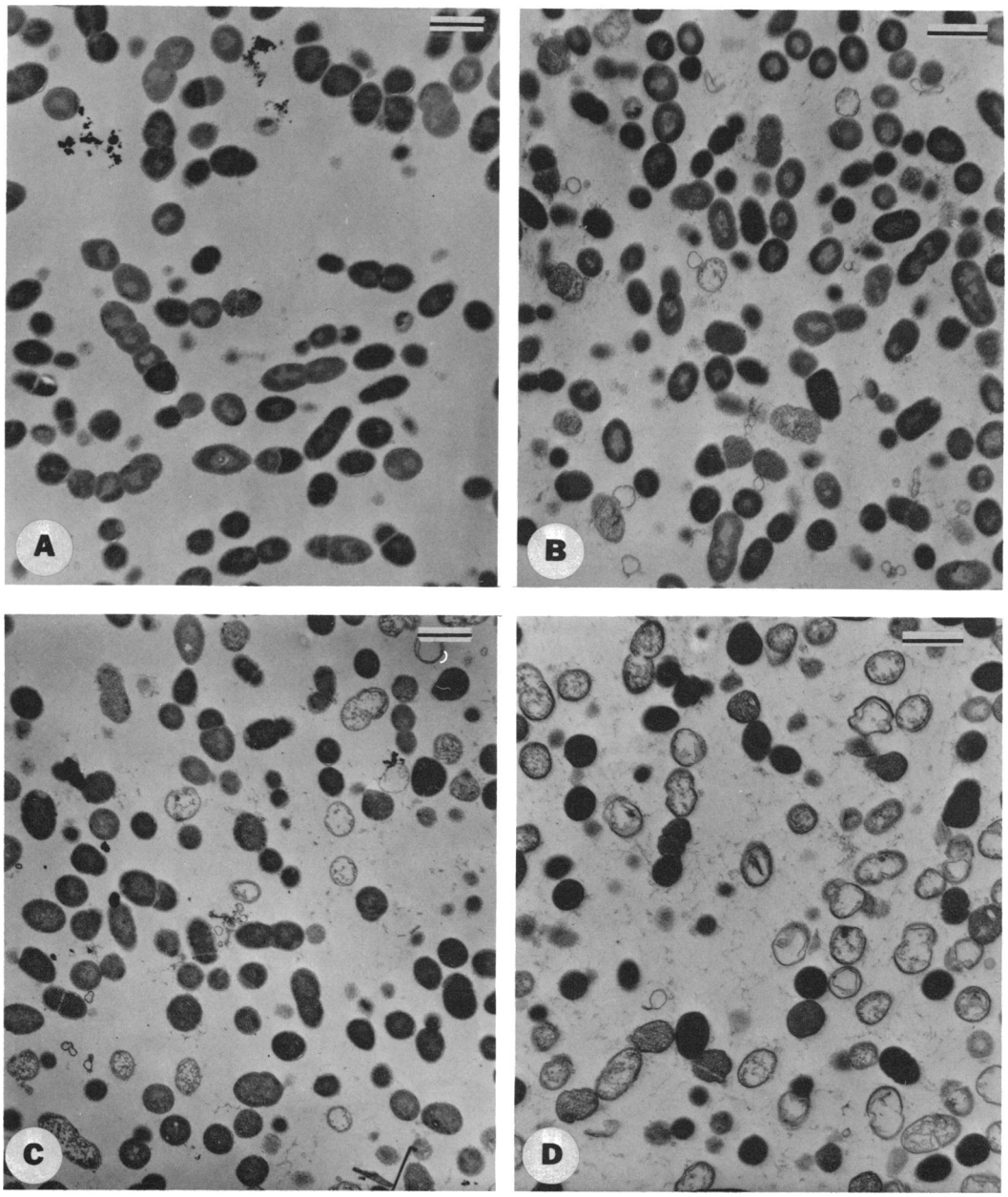
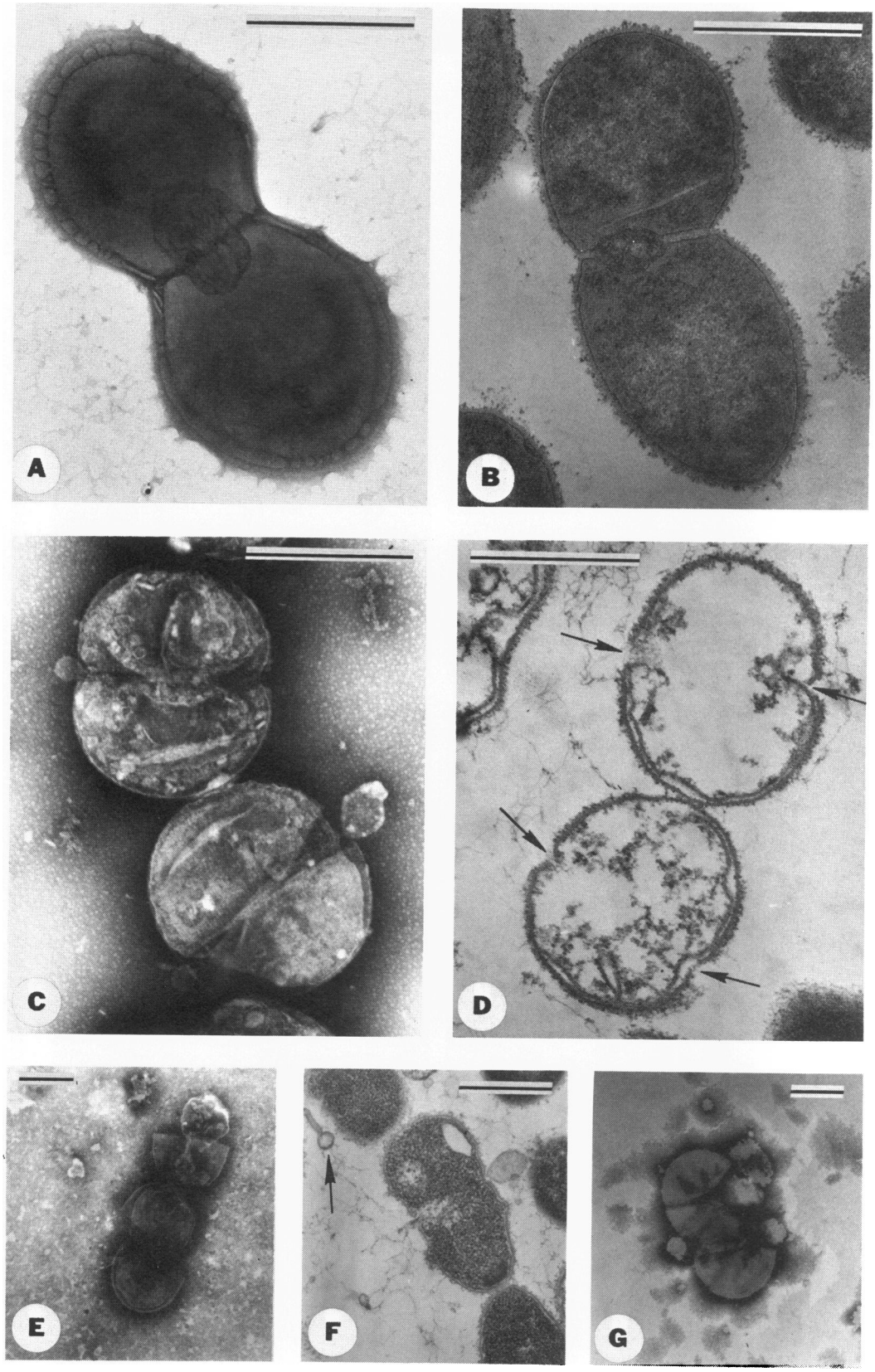


FIG. 8. *Ultrathin sections of competent Wicky culture from lysing buffer at 37 C. Samples taken at (A) 0, (B) 4, (C) 10, and (D) 30 min. Marker bars equal 1.0 μm.*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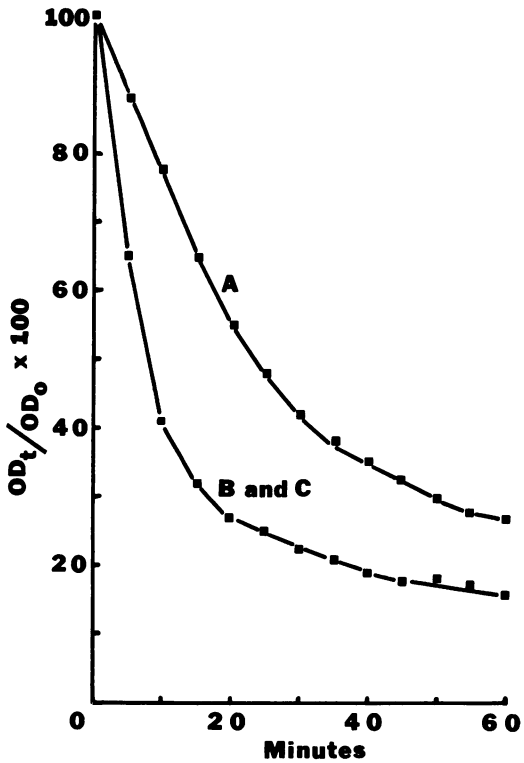

FIG. 10. *Effect of native and alkali-denatured transforming DNA on the rate of autolysis of competent Wicky cells. Wicky cells were made competent by treating them for 20 min with competence-factor. At 20 min, either no (B), native (A), or alkali-denatured (C) DNA was added (both at 8 μg/ml) to each of the three cultures. The cultures were incubated for an additional 30 min before harvesting for lysis. The remainder of the protocol followed that as already described.*

viable), 67% of the potential transformants were lost; when 67% of the culture lysed (32% of the cells still viable), 79% of the potential transformants were lost. This rapid loss of potential transformants shows that competent cells are lysing.

DISCUSSION

The role of autolysins in competence development is not yet fully substantiated. Young and Spizizen (29) showed that the cell walls of competent cells of *B. subtilis* contained more lytic activity than cell walls prepared from noncompetent cells. However, both had lytic activity. Stewart and Marmur (27) showed that the extractable autolysin from competent *B. subtilis* cells appeared maximally 30 min after the cells were treated with DNA. In this case, only competent cells had autolysin, and heterologous DNA functioned as well as homologous DNA. In addi-

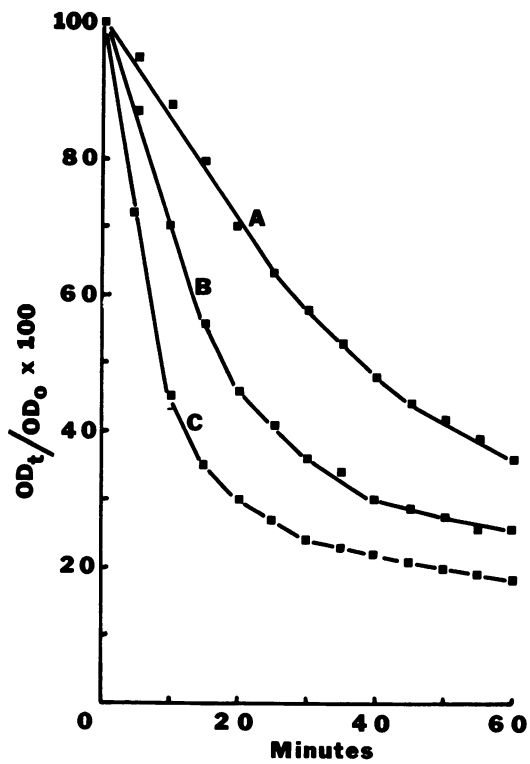

FIG. 11. *Effect of native and alkali denatured calf thymus DNA on the rate of autolysis of competent Wicky cells. Wicky cells were made competent by treating them for 20 min with competence-factor. At 20 min, either no (C), native (A), or alkali-denatured (B) DNA was added (both at 8 μg/ml) to each of the three cultures. The cultures were incubated for an additional 30 min before harvesting for lysis. The remainder of the protocol followed that as already described.*

FIG. 9. *Intact (noncompetent) and lysed (competent) cells of group H streptococci. All marker bars equal 0.5 μm. (A) Intact noncompetent cells of strain Wicky from lysing buffer at 37 C; negatively stained. The same appearance is seen in noncompetent Challis in the buffer, precompetent cells in buffer, competent or noncompetent cells in culture media, or competent cells in lysing buffer at 0 C. (B) Same as part A; in ultrathin section. (C) Competent Wicky cells in lysing buffer at 37 C for 30 min; negatively stained. Competent Challis cells appear the same. (D) Same as part C; in ultrathin section. Note minimal equatorial gaps in cell wall (arrows), retention of cytoplasmic membrane, and strands of extruded DNA. (E and G) Competent Wicky cells after 24 hr in lysing buffer at 37 C; negatively stained. Note cell wall hemispheres, with no further degradation, and retained cytoplasmic membrane. (F) Ultrathin section of competent Wicky cell from lysing buffer after 10 min at 37 C. Stage of early lysis, showing extruded protoplasmic bleb, extruding DNA strands at equator of cell, and extruded mesosome fragment (arrow).*

TABLE 1. *Effect of native and alkali-denatured DNA on transformation and autolysis of Wicky cells*[a]

DNA (8 μg/ml) added to Wicky cells	Time for 50% reduction in optical density (min)	Transformation (%)	Inhibition of transformation (%)
Expt 1			
None	9	11.6[b]	0
Native calf thymus	37	3.2[c]	72
Denatured calf thymus	18	5.6[c]	52
Expt 2			
None	8	7.8[b]	0
Native transforming	23	9.4[d]	0
Denatured transforming	8	0.12[d]	99
Denatured transforming		9.7[e]	0

[a] Wicky cells were grown in BHI-HS for 40 min and treated with CF for 20 min at 37 C. DNA when present was added for 30 min.

[b] Cells at 30 min were treated with transforming DNA for an additional 30 min.

[c] Cells first treated for 30 min with calf thymus DNA were exposed to transforming DNA for an additional 30 min.

[d] Cells exposed for 30 min to transforming DNA were incubated an additional 30 min in the absence of DNA.

[e] Cells exposed for 30 min to alkali-denatured transforming DNA were exposed an additional 30 min to native transforming DNA. Harvesting cells for lysis was done 50 min after the addition of CF.

tion, the appearance of autolysin was sensitive to antibiotics (CAP and cellocidin). These latter authors suggest that incoming DNA damages the cell wall, and, therefore, the extractable autolysin may be involved with cell wall repair mechanisms. In pneumococci, the role played by autolysins in the development of competence is uncertain. Lacks (10) showed that mutant cells that are autolysin negative still develop competence. However, pneumococcal cells that have ethanolamine in place of choline in their cell wall teichoic acid are unable to lyse and cannot become competent (28).

The data presented in this report do show a correlation between cell autolysin activity and competence development: only competent cells autolyze. Although not established precisely, it does appear that the rate of autolysis is roughly proportional to the level of transformation (Fig. 2). Challis cultures that transformed with a frequency of 0.05% took 60 min for 50% lysis, whereas cultures that transformed with a frequency of 1.9% took 45 min. Wicky cells that transformed with a frequency of 6 to 8% lysed to 50% in 6 to 8 min. A better parameter that might show a direct correlation between autolysis rate and competence may be population competence and not frequencies of transformation to single markers. Wicky cells are ideal for this type of study since population competence can be experimentally controlled by limiting the amount of CF (17).

The autolysin in competent streptococcal cells is active at basic *p*H values and is stimulated by reducing agents. Competent Wicky cells suspended in only 0.02 M 2-ME (*p*H 5 to 6) or in 0.05 M TAPS buffer containing 0.02 M 2-ME (*p*H 4.8) do not autolyze at 37 C. On the other hand, such cells suspended in TAPS buffer (*p*H 9.0) without 2-ME lyse as shown in Fig. 1, curve C; the lysis, however, is less rapid and less extensive than in the presence of 2-ME (Fig. 1, curve B). Therefore, a combination of high *p*H and reduced conditions appears optimal. Divalent cations, such as calcium and magnesium (tested at 10^{-3} M and 3×10^{-2} M, respectively), retarded the rate of autolysis. It also appears that nongrowth conditions must prevail to observe autolytic activity. Addition of 2-ME to competent Wicky cells in BHI-HS (*p*H 7.1 to 7:4) did not cause a reduction in viable cell titer nor in the number of transformations. Similar cells in buffer containing 2-ME (*p*H 7.1 to 7.4) lysed. Lysis, therefore, may reflect an activated autolysin (cell wall-synthesizing enzyme?) under conditions of unbalanced growth.

Although autolysis occurs in group H streptococcal cells after the action of CF, we do not yet know whether CF activates an autolysin that is already there, whether CF inhibits cell wall biosynthesis and thus allows the "normal" autolytic activity that is involved in wall growth (24, 31) to become predominant and excessive, whether CF induces an activator for the autolysin [a protein activator may be involved in the conversion of latent autolysin to active autolysin in *S. faecalis* (19)] or induces the autolysin de novo. The last two possibilities seem more plausible, since protein synthesis is required for the expression of CF (as competence) and the expression of the autolysin.

The electron microscopic observations verify lysis of competent cells as the cause of decreasing OD in lysing buffer and establish that lysis is limited to the equatorial region representing the youngest (5, 7) wall and cross-wall. Since it is increasingly apparent that cell wall replication is the result of alternating biosynthetic and autolytic activities (26), and since it was shown that autolysis in another *Streptococcus* is initiated at the cross-wall (6) which represents most newly formed cross-wall (7, 25), it is not surprising that

autolysis in group H streptococci—however initiated—should also be first demonstrable at the cross-wall region. Such equatorial lysis was previously observed (8, 16, 26). However, in this study we have not seen evidence of enzymatic dissolution of "the leading edge of the nascent cross-wall" (6) nor of any continued attack on adjacent peripheral wall such as described by Shockman and Martin (26). The reasons for apparent limitation of autolysis to the equatorial plane in competent group H streptococci under the described conditions are unknown. It is possible that only an active autolysin, as opposed to a latent one, is involved, as in *S. faecalis* ATCC 9790, and that "old" wall is protected in a fashion analogous to the protection of *M. lysodeikticus* walls from lysozyme by *O*-acetylation in the glycan chains of the mucopeptide (4), or both. However, in the absence of detailed knowledge of the structural chemistry of the group H streptococcal cell wall or mucopeptide or of the nature of the autolysin, no meaningful speculation can be made.

The effect of DNA on the rate of autolysis is not yet understood. Unlike the enzyme in *B. subtilis* (27), the streptococcal autolysin is retarded in activity, not enhanced, by DNA. There appears to be a correlation between the reactivity of the DNA with competent Wicky cells and the rate of autolysis: the more the DNA interferes with transformation (or promotes it) the greater the time required for 50% reduction in OD (Table 1). Possibly, the DNA blocks the enzyme substrate or modifies it in some unknown way. The reason alkali-denatured calf thymus DNA did not bring the autolytic rate back to the control value is unknown. Possibly, calf thymus DNA is more cross-linked than transforming DNA or differs in molecular weight. Experiments are in progress to test these ideas.

The relationship of the newly appearing competence-associated protein (20) to the autolysin or to autolysin activation is also currently under study.

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