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Biochemical and Cellular Changes Occurring During Conjugation in *Hansenula wingei*

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ABSTRACT

BROCK, THOMAS D. (Indiana University, Bloomington). Biochemical and cellular changes occurring during conjugation in *Hansenula wingei*. J. Bacteriol. **90**:1019–1025. 1954.—A technique has been devised for deagglutinating mixed populations of conjugating cells so as to be able to visualize microscopically early stages of the conjugation process. A cell can form a conjugation tube only when in contact with a cell of opposite mating type, but may do so even if the mate is unresponsive or ultraviolet-inactivated. Cell fusion occurs, however, only when both cells are able to form conjugation tubes in a region of contact. Fusion begins almost as soon as the two cells begin to form protuberances, and long before any dissolution of cell-wall material between the cells occurs. A cell which has conjugated in one region of its cell wall is still able to conjugate with another cell in another region, so that triply and quadruply conjugated cells are occasionally formed. There is no significant *net* increase in deoxyribonucleic acid, ribonucleic acid, protein, or carbohydrate which might be related to the conjugation process, because any minor changes that occur in these components are also detected when cells of only one mating type are incubated or when the conjugation process is inhibited with the antibiotic cycloheximide. Changes in activity of β-1,3-glucanase (with laminarin as substrate) and β-1,6-glucanase (with pustulan as substrate) have been measured during the conjugation process, in addition to changes in the activity of several control enzymes which would not be expected to be related to the conjugation process. Significant increases in invertase (sucrase), laminarinase, and pustulanase were detected, and minimal increases occurred in β-glucosidase and acid phosphatase. However, these same increases were also observed in controls involving only one mating type; thus, these increases are probably not related to the conjugation process, but may be a result of other processes which probably occur during incubation in the conjugation medium.

The sexually agglutinative yeast *Hansenula wingei* is a favorable organism for the study of conjugation, because the strong attraction between mating types ensures that a high percentage of the cells fuse. Earlier work on the physiology of conjugation (Brock, 1961) showed that both mating types must function and synthesize protein in order for conjugation to take place. However, in that work only the formation of mature conjugants was studied, and the early stages of the process were not examined. In fact, the massive agglutination has made the light microscopic analysis of the conjugation process difficult, because, until recently, the only way of deagglutinating the cells involved lethal heat treatments. In the first part of the present paper will be presented a simple technique for deagglutination which does not kill the cells; this technique was used to study the early events of the fusion process. From this study it is concluded that the interaction between two cells is strictly a localized event involving the region where the cells touch, and that a given cell can occasionally fuse with more than one other cell. Another conclusion is that cell fusion begins very early in the conjugation process, almost at the moment when the cell walls begin to expand, but that fusion occurs only if the cell walls of both cells are sending out conjugation tubes at the same place. A single cell may send out a conjugation tube in the absence of a similar process in its mate, but fusion then does not take place.

Because the earlier work (Brock, 1961) had shown the involvement of protein synthesis during the cell fusion process, experiments have been performed on changes in macromolecular composition during conjugation. For such experiments to have meaning, it would be essential that macromolecular changes unrelated to conjugation should not occur. Because the conjugation me-

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dium is devoid of nitrogen sources and essential vitamins, and because it had been shown earlier that cells of a single mating type suspended in such a medium do not bud or increase in cell number, it seemed likely that any macromolecular changes which might be detected would be related to the conjugation process. The present work will show, however, that no significant changes related to conjugation can be detected in these constituents; indeed such changes probably would not be expected to be measured, since the conjugation process is strictly localized in character, involving only a small portion of the protoplasmic mass of each cell. However, in the later stages of the fusion process, a considerable amount of cell-wall dissolution occurs, as shown by electron microscopy (Conti and Brock, 1965), so that it seemed likely that, even though total macromolecular changes might not be detected, the synthesis of specific enzymes, which are responsible for this dissolution, might be detected. Changes in the activity of two enzymes, β-1,3- and β-1,6-glucanase, which are probably involved in the wall dissolution process, were therefore studied. It will be shown that, although significant increases in these enzymes do occur in a manner correlated with the conjugation process, similar increases occur when cells of one mating type are incubated alone in conjugation medium.

The present work thus further defines the nature of the conjugation process, and reveals some of the problems attendant to any examination of a morphogenetic event at the biochemical level.

MATERIALS AND METHODS

Cultures. H. wingei NRRL Y-2340, strains 5 and 21, the agglutinative mating types, were obtained in 1957 from L. J. Wickerham, and they have been kept in vegetative culture since then with no diminution in their sexual powers.

Growth and conjugation. The cells were grown into the stationary phase (2×10^8 to 4×10^8 per milliliter) on a rotary shaker at 30 C in a liquid medium of the following composition: glucose, 30 g; yeast extract (Difco), 7 g; KH_2PO_4, 5 g; water, 1,000 ml. The cells were then washed twice in water and suspended in water, and the cell densities of the two strains were adjusted to be equal. The two suspensions were then mixed, 0.1 volume of 1% $MgSO_4 \cdot 7H_2O$ was added, the mixture was centrifuged in a clinical centrifuge, and the agglutinated cell pack was suspended to give a final cell concentration (as unagglutinated cells) of about 3×10^8 per milliliter in the conjugation medium: 0.01 M KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.5% glucose, at *p*H 5.5. The agglutinated suspension was then placed on a rotary shaker at 30 C, and samples were removed at various intervals.

Conjugation assay. The routine assay involved deagglutinating the cells in an autoclave for 15 min at 121 C in 8 M urea (Brock, 1961) followed by a differential count of conjugated and unconjugated cells. Such an assay lumps together various stages of conjugated cells, and thus does not distinguish between early and late stages of the process. The special deagglutination procedure developed for the study of the early events is described in the section on results.

Macromolecular assays. Samples (5 ml) of the suspension were removed at intervals, washed twice in water, treated for 1 hr in the cold with 5 ml of 0.5 N perchloric acid, and centrifuged; the supernatant fraction was discarded, and the pellet was suspended in 5 ml of 0.5 N perchloric acid and heated at 100 C for 20 min. The suspension was then centrifuged and the supernatant liquid was used for ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) assays by means of the orcinol (Dische, 1955) and diphenylamine (Burton, 1956) methods, respectively. The pellet of the hot perchloric acid treatment was suspended in 5 ml of 1 N NaOH, heated for 20 min at 100 C, and assayed for protein by the procedure of Lowry et al. (1951).

Carbohydrate assays. Carbohydrate measurements were performed on 13-ml samples taken in parallel with the above samples, and fractionated and assayed by the method of Trevelyan and Harrison (1952).

Cell-free extracts. In parallel with the above, 140-ml samples were taken and were washed twice in 0.01 M potassium phosphate (*p*H 5.5); the final pellet was frozen at −70 C. Each frozen sample was then ground in the cold for 5 to 10 min with approximately two times its volume of alumina (Buehler levigated alumina 1557 AB). The ground pellets were suspended in 10 ml of cold 0.01 M potassium phosphate (*p*H 5.5) and centrifuged at $39,000 \times g$ for 10 min, and the supernatant fluid was used for enzyme assays. Because *H. wingei* cells are difficult to break, a variety of procedures were tried, including sonic treatment and breakage with glass beads. In control experiments the bulk of the β-glucanase activity was found to be in the supernatant fraction; consequently, only this fraction has been assayed. Since the yeast cells might be expected to vary in sensitivity to breakage in different stages of the conjugation process, the precise degree of breakage was estimated in each case by an assay for protein on the $39,000 \times g$ supernatant fraction, and the enzyme activities in the extracts have been converted to specific activities. Actually, no differential sensitivity to breakage was observed, and most extracts contained approximately the same amount of protein.

Enzyme assays. Some general properties of yeast β-glucanase have been reported elsewhere (Brock, 1965). One volume of extract at *p*H 5.5 was incubated at 30 C in a total volume twice its amount with 1 mg/ml of substrate. At intervals, 0.5-ml samples were deproteinized and assayed for reducing sugar by the Nelson-Somogyi method (Hawk, Oser, and Summerson, 1954). The rate of release of glucose was linear with time for at least 2 hr, and the rate of release was proportional to

enzyme concentration. In the case of cellobiose, hydrolysis was measured by the release of glucose as assayed by use of the Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). When *p*-nitrophenyl-β-D-glucoside (PNPG) hydrolysis was to be measured, 0.5-ml samples were taken from the incubation mixture, diluted with 4.5 ml of 4% Na_2CO_3, and the absorption was read at 420 mμ. Enzyme activity is defined as milligrams of glucose or of *p*-nitrophenol released per hour per milligram of protein. Acid phosphatase was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate in 0.01 M potassium phosphate (*p*H 5.5). The pustulan was a gift of E. T. Reese; all other substrates were commercial preparations.

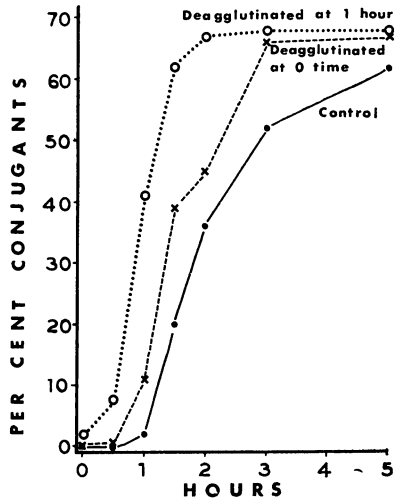
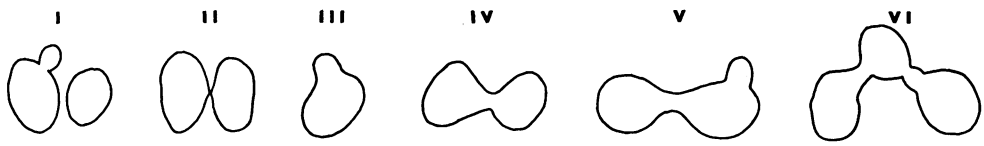


FIG. 1. *Time course of conjugation in suspensions which have been deagglutinated at times indicated and then immediately reagglutinated. Incubation in all cases began at same time. Time plotted is that after reagglutination.*

RESULTS AND DISCUSSION

Deagglutination procedure. This procedure is based on the fact that no agglutination between mating types occurs when the cells are suspended in distilled water (Brock, 1958). A sample of agglutinated cells was taken at an appropriate time and washed twice in distilled water. Although this procedure broke up most of the large clumps, many small clumps remained, which were then broken up by treatment of the suspension for 10 sec at full power (rated by the manufacturer at 75 w average) in a Sonifier (Branson Instrument Inc., Stamford, Conn.). This treatment has no detectable effect on cell structure or viability, as this yeast is so highly resistant to sonic treatment that 5 min under the same condition is required to obtain about 50% cell breakage. Once deagglutinated, the cells remain unclumped for many hours or days in distilled water, although they will reagglutinate immediately if they are made 0.1% in $MgSO_4 \cdot 7H_2O$.

Deagglutinated cells are able to begin conjugation immediately if they are suspended in conjugation medium. Figure 1 shows a time course of the conjugation process with cells which were not deagglutinated, cells which were deagglutinated immediately after mixing, and cells which were deagglutinated after 1 hr of incubation. As can be seen, in the control there was approximately a 1-hr lag before conjugation began. With cells deagglutinated immediately after mixing (and



	I	II	III	IV	V	VI
0 hr	100	0	0	0	0	0
0.5	100	0	0	0	0	0
1	100	0	0	0	0	0
1.5	77	17	3	3	0	0
2	75	9	6	8	0	2
3	42	5	12	32	0	9
4	31	5	3	50	6	5
5	20	8	5	62	3	2

FIG. 2. *Differential counts of various classes of conjugants, in percentage of total cells counted. (I) Single cells and cells with buds. (II) Early stages of fusion. (III) Cells with protuberances. (IV) Simple conjugants. (V) Conjugants with protuberances. (VI) Triply and quadruply conjugated cells.*

then reagglutinated), the lag was reduced to 30 min. Cells incubated for 1 hr and then deagglutinated, at which time the lag should have been over, began conjugating immediately upon reagglutination. Viable counts of deagglutinated suspensions were identical to the counts of control suspensions which had never been agglutinated. Thus, the deagglutination procedure institutes no detectable cellular damage.

Microscopic observations. Earlier microscopic observations on the conjugation process (Brock, 1961) were made in microculture in which pairs of cells were isolated and allowed to interact. However, in agglutinated populations, large numbers of cells are thrown together, and any cell is given several or many other cells with which it may conjugate. An analysis of conjugation in such situations reveals new information about the control of the fusion process. If deagglutinated populations are examined carefully under a phase microscope at various times of incubation, several stages of conjugation can be seen. Figure 2 summarizes an experiment in which various categories of cells were counted after various incubation times.

When agglutinated populations are deagglutinated by sonic treatment immediately after mixing, before any incubation period, only single cells are seen and, of hundreds of cells examined, no cells have ever been seen which were attached to each other. Within 1 or 2 hr of incubation (depending on the experiment), some cells are paired, but with only a very slight protuberance between them (type II in Fig. 2). Note that even though the cell-wall deformation is minimal, the cells have already interacted in such a way that they do not come apart in the deagglutination procedure. Presumably, fusion of cell-wall material has already occurred, even at this early stage. Attempts to induce such fused cells to separate by treatment with 0.25 M β-mercaptoethanol or 1 mg/ml of chymotrypsin, agents which destroy the specific mating substances of strains 5 and 21, respectively (Brock, 1959; Taylor, 1964), have been unsuccessful. As conjugation proceeds, the protuberances connecting the two cells progressively elongate, until the conjugation tube is fully formed (type IV of Fig. 2).

Not all cells which begin to send out a protuberance find a responsive mate. If the adjacent cell does not respond, the protuberance may continue to grow, but no fusion occurs. In deagglutinated suspensions, as many as 10% of the single cells may show protuberances, especially in samples from later incubation times. The distinction between a bud and a protuberance is shown in Fig. 2. A bud always shows a pronounced constriction at the base, even when quite large, whereas a protuberance has a broad base and hence resembles a conjugation tube. Such protuberances are never seen when populations of only one mating type are incubated under the same conditions. Thus, the existence of such protuberances reveals that a cell in an agglutinated suspension can be induced to respond by the presence of the opposite mating type, even when the mate is unresponsive in return.

If the cells of one of the mating types are inactivated by ultraviolet light before the two populations are mixed, no conjugants form (Brock, 1961). However, the nonirradiated mating type is still able to respond to the presence of its mate and form protuberances, without the occurrence of cell fusion. To reveal this process, microcultures were set up on agar blocks of conjugation medium, in which only one of the mating types was irradiated. After incubation for 4 hr, a thin slice of the agar containing the cells was carefully removed, placed on a slide, and covered gently with a cover slip, so as not to disturb the orientation of the cells. A number of pairs were seen in which one cell had sent out a protuberance in the region where the two cells had touched. Never in such cases was a protuberance directed away from the inactivated cell, further showing that cell contact is in some way involved in the control of the growth process of the conjugation tube. In earlier work (Brock, 1961), it was stated that ultraviolet-irradiated cells induce nonirradiated cells of the opposite type to bud. With the more precise microscopy available at present, it can be concluded that in most cases the structures formed are not buds, but protuberances.

After 3 to 4 hr of incubation, over 50% of the cells in an agglutinated population have conjugated, and in the large majority of cases the conjugants consist of only two cells. However, in about 1 to 10% of the cases, three cells are seen in conjugation (type VI in Fig. 2). Rarely, four cells are seen in conjugation, usually in such a way that two cells of adjacent conjugal pairs have fused. These results show that when a cell has begun to conjugate in one region of its cell wall it is not prevented from conjugating in other regions. Therefore, the conjugation response appears to be strictly a localized event of a small region of the cell wall. On some occasions, a conjugant is seen with an additional protuberance (type V of Fig. 2), and presumably this situation arises when a conjugant begins to respond to the presence of another adjacent cell, but finds this other cell unresponsive.

Cell contact is required for the conjugation process. This has been shown by preparing agar blocks of conjugation medium on which mixtures of cells were placed at such densities that many

of the cells were close together but were not touching. Only when two cells were actually touching did conjugation take place. In another experiment, two agar bocks were separated by a membrane filter; a dense suspension of one mating type was placed on one block, and a dilute suspension on the other. No evidence for the formation of protuberances could be found under these conditions. The requirement for cell contact may exist only in *H. wingei* (and possibly other sexually agglutinative yeasts), because Levi (1956) has shown that baker's yeast cells can respond to mates which are not touching.

Macromolecular changes. In a large number of experiments, samples of conjugating suspensions or control suspensions of only one mating type were sampled at various time periods, and assays for various macromolecular constituents were performed. In general, there were only slight changes during a 5-hr time period in which over 70% of the cells conjugated. Table 1 provides a summary of one of these experiments, which involved not only a conjugating suspension, but a parallel flask in which conjugation was inhibited by 3 μg/ml of cycloheximide, and other flasks with separate suspensions of the unmixed mating types. It can be seen that there were only minimal changes, mostly within experimental error, of the contents of DNA, RNA, protein, total carbohydrate, glucan, and glycogen. Mannan content decreased significantly in all cases, but mannan is only a small fraction of the total carbohydrate, and it decreased to a marked degree in the controls as well as in the conjugating suspension.

Thus, it can be concluded that, during conjugation, it is not possible to detect any conjugation-specific changes in macromolecular constituents.

Enzyme changes. Although no net changes in macromolecules could be detected, it was felt that changes in specific enzymes, which would represent only a small portion of the total protein of the cell, might still be found. Because of the extensive alterations of the cell-wall structure during conjugation, attention was focused on β-glucanases, which might act on the rigid glucan layer. A general review of the enzymatic hydrolysis of β-glucans was presented by Reese and Mandels (1963). Unconjugated cells of *H. wingei* possess intracellular soluble β-1,3- and β-1,6-glucanases, which act on the substrates laminarin

TABLE 1. *Changes in macromolecular constituents during 5 hr of incubation (multiples of initial values)**

Constituent	Strain 5 alone	Strain 21 alone	Mixture (74% conjugants)	Mixture in 3 μg/ml of cycloheximide (0% conjugants)
DNA	0.82 (22)	1.1 (20)	1.3 (24)	1.1 (25)
RNA	1.0 (600)	0.94 (610)	0.96 (670)	1.0 (630)
Protein	1.0 (770)	1.0 (690)	0.96 (730)	0.92 (610)
Total carbohydrate	1.0 (1,400)	1.0 (1,200)	1.1 (1,400)	1.0 (1,800)
Glucan	1.5 (400)	1.0 (302)	1.5 (312)	1.4 (316)
Glycogen	1.5 (560)	1.7 (480)	1.2 (555)	1.4 (570)
Mannan	0.58 (43)	0.12 (43)	0.33 (45)	0.45 (45)
Cell count	2.3 (3.3)	1.5 (3.6)	1.5 (3.2)	1.5 (3.0)

* The initial value for each constituent, in micrograms per milliliter of cell suspension, is given in parentheses. The initial value for cell count in parentheses should be multiplied times 10^8 per milliliter.

TABLE 2. *Increase in specific activity of various hydrolytic enzymes after 5 hr of incubation (multiples of initial values)**

Substrate	Strain 5 alone	Strain 21 alone	Mixture (72% conjugants)
p-Nitrophenyl-β-D-glucoside	2.5 (142)	1.7 (155)	1.9 (250)
p-Nitrophenyl phosphate	2.4 (10)	2.3 (3.9)	1.0 (13)
Laminarin	3.2 (6.8)	2.2 (4.1)	4.5 (8.3)
Pustulan	3.2 (0.99)	2.9 (0.63)	3.5 (1.3)
Sucrose	13 (0.52)	14 (0.17)	21 (0.35)
Cellobiose	2.5 (2.1)	2.5 (1.1)	2.5 (2.8)

* The initial value for each constituent, in micrograms per milligram of protein per hour, is given in parentheses.

and pustulan, respectively. These enzymes are exo-hydrolases, acting at the end of the polysaccharide chain and splitting off only free glucose, as shown by paper chromatography. No endo-hydrolase is found, as shown by the absence of any detectable activity against the mixed β-1,3–β-1,4-glucan, lichenin. A more detailed discussion of the nature of yeast β-glucanases has been presented elsewhere (Brock, 1965). It has been shown that in baker's yeast probably a single enzyme acts on both β-1,3 and β-1,6 linkages, with about equal facility. In *H. wingei*, the β-1,6 activity is less rapid than in baker's yeast, and the existence of the two activities in one enzyme has not been definitely shown. The purified yeast β-glucanases also act on the chromogenic substrate PNPG, although not on phenyl-β-D-glucoside or other aryl- or alkyl-glucosides. However, PNPG is also the classical substrate for β-glucosidase, and *H. wingei* has a highly active β-glucosidase which does not act on laminarin or pustulan, but which does act on cellobiose and PNPG. It has been possible to separate the β-glucosidase and β-glucanase of *H. wingei* on diethylaminoethyl cellulose columns, and both enzymes hydrolyze PNPG, but only the β-glucanase hydrolyzes laminarin and pustulan (Brock, 1965). Thus, an assay in crude extracts with laminarin as a substrate is specific for β-glucanase. On the other hand, the β-glucosidase activity in crude extracts against PNPG is so high at *p*H 5.5 to 6.0 that it masks any β-glucanase activity on this substrate, so that with PNPG as substrate the assay is essentially specific for β-glucosidase. At *p*H 4, the β-glucosidase is inactive, and all *p*-nitrophenyl-β-D-glucosidase activity at this *p*H is due to β-glucanase, so that a differential assay is possible. However, in the present work β-glucanase has been assayed by the hydrolysis of laminarin and pustulan, to ensure that assays in crude extracts directly reflected the enzyme of interest.

A variety of experiments have been done to measure changes in specific activity of β-glucanase, as well as other hydrolytic activities, at various times during the conjugation process. In addition, control suspensions of each mating type alone have been studied under the same conditions. In general, the increases in eznyme activity which have been detected occurred progressively over the incubation period, and the time courses for these changes present no significant data; therefore, results are presented only for a 5-hr incubation period, at which time over 70% of the cells in a mixed suspension had conjugated.

Table 2 shows that all of the hydrolytic enzymes measured increase in specific activity to various degrees during the time period. The most striking increases are noted for invertase (sucrase), laminarinase, and pustulanase. Although the enzyme increases are definitely higher in the conjugating cells than in the control suspension, increases do occur in the latter which are definitely greater than variations due to experimental error. It seems likely that these increases in enzyme activity are unrelated to conjugation, and a more reasonable interpretation is that they are a result of processes which occur during the 5-hr incubation period in the nitrogen-deficient conjugation medium. In this respect, it should be recalled that the conjugating suspension is highly agglutinated, whereas the control suspensions are not, so that the controls and the conjugating suspensions are not strictly comparable. Indeed, I have shown by use of radioactive amino acids and pyrimidines that agglutinated suspensions take up these substances considerably less efficiently than do control cultures, and it is likely that these agglutinated suspensions are more impermeable to glucose and oxygen as well. Thus, autolytic processes might be expected to be more pronounced in such relatively dense suspensions in any event, and it seems likely that the minimal differences between control and experimental cultures can be explained on these grounds.

The increases in enzyme activity, even though not related to conjugation, are of some interest, especially when it is recalled that there is no net increase in protein during the incubation period (Table 1). Especially the 13- to 20-fold increase in sucrase activity seems worthy of further study.

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