JOURNAL OF BACTERIOLOGY, Nov. 1995, p. 6625–6629 Vol. 177, No. 22
0021-9193/95/$04.00+0
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Ultrastructural Diversity of the Cellulase Complexes of *Clostridium papyrosolvens* C7

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Received 8 June 1995/Accepted 30 August 1995

Transmission electron microscopy was used to investigate the ultrastructural features of diverse cellulase and cellulase-xylanase multiprotein complexes that are components of the cellulase-xylanase system of *Clostridium papyrosolvens* C7. The multiprotein complexes were separated by anion-exchange chromatography into seven biochemically distinguishable fractions (F1 to F7). Most individual F fractions contained, in relatively large numbers, an ultrastructurally recognizable type of particle that occurred only in smaller numbers, or not at all, in the other F fractions. It is suggested that these ultrastructurally distinct particles represent the biochemically distinct multiprotein complexes that constitute the cellulase-xylanase system of *C. papyrosolvens* C7. Some of the particles consisted of tightly packed globular components that appeared to be arranged in the shape of a ring with conical structures pointing out along its axis. Other particles had triangular, polyhedral, or star shapes. The major protein fraction (F4) almost exclusively contained particles consisting of loosely aggregated components, many of which appeared to be arranged along filamentous structures. The ultrastructural observations reported here support our previous conclusion that the cellulase-xylanase system of *C. papyrosolvens* C7 comprises at least seven different high-molecular-weight multiprotein complexes. Furthermore, results of this and earlier studies indicate that the interactions between *C. papyrosolvens* C7 and cellulose are different from those that have been described for *Clostridium thermocellum*.

The extracellular cellulase-xylanase system of *Clostridium papyrosolvens* C7 comprises at least seven distinct high-molecular-weight multiprotein complexes (M_r, 500,000 to 660,000), each with different polypeptide compositions and enzymatic properties (16). All seven multiprotein complexes have endoglucanase-active protein subunits, but only two of the complexes have xylanase-active subunits (16). Three of the multiprotein complexes hydrolyze crystalline cellulose (i.e., have Avicelase activity) (16). Arabinofuranosidase, β-xylosidase, and acetylesterase activities were detected in cellulase- and xylanase-active complexes (17). Hydrolysis of crystalline cellulose by *C. papyrosolvens* C7 involves synergistic interactions in which the diverse multiprotein complexes participate (16).

Cellulolytic bacteria, such as *C. papyrosolvens* C7, inhabit natural environments in which cellulose is present primarily in plant cell walls in association with other polysaccharides or polymers (e.g., xylan and other hemicellulose components) (13, 16). Generally, anaerobic bacteria that degrade plant cell wall material synthesize extracellular multiprotein complexes that function in the concurrent hydrolysis of different plant polysaccharides. The cellulase-xylanase system of *C. papyrosolvens* C7 differs from previously described cellulose-hydrolyzing systems of other anaerobic bacteria in that it includes diverse multiprotein complexes, i.e., it is a multicomplex rather than a unicomplex system.

In the present study we used transmission electron microscopy to investigate the ultrastructural features of individual cellulase and cellulase-xylanase multiprotein complexes that are components of the cellulase-xylanase system of *C. papyrosolvens* C7. The results we obtained support our previous conclusion that *C. papyrosolvens* C7 utilizes a multicomplex cellulase-xylanase system for the hydrolysis of cellulose.

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MATERIALS AND METHODS

Bacterial strain, culture conditions, and enzyme assays. *C. papyrosolvens* C7 (formerly *Clostridium* strain C7) (13) was cultured in the chemically defined medium MJ-CB as previously described (4, 16). Procedures used in determining Avicelase, carboxymethylcellulase (CMCase), and xylanase activities, and in assaying protein, were described previously (16). Avicel, type PH 105, in the form of 20-μm particles (FMC Corp., Marcus Hook, Pa.), was the substrate in the Avicelase assay. The Avicelase assay measures the ability of cellulase preparations to hydrolyze crystalline cellulose (4). The CMCase assay is used to measure endo-1,4-β-glucanase activity.

Gel filtration and anion-exchange chromatography. Ultrafiltration of supernatant fluid from late-log-phase *C. papyrosolvens* cultures, isolation of the multicomplex preparation by gel filtration chromatography of concentrated supernatant fluid, and separation of fractions F1 through F7 by anion-exchange chromatography of the multicomplex preparation were described previously (16).

Enzyme preparations were stored in succinate buffer (0.1 M succinate-NaOH, pH 6.0, containing 0.05 M NaCl and 0.025%, wt/vol, sodium azide) or in 0.05 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 7. Samples were kept at −20 or 4°C.

PAGE. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) in the presence and absence of sodium dodecyl sulfate (SDS), using a PhastSystem and PhastGels (4 to 15% polyacrylamide linear gradient; Pharmacia, Uppsala, Sweden). Glycerol (20%, vol/vol, final concentration) was added to samples before they were applied to nondenaturing (native) polyacrylamide gels. Prior to SDS-PAGE, samples were boiled in sample buffer (10) for 5 min. A fraction obtained following anion-exchange chromatography (fraction F5; see Results) was incubated in sample buffer that contained 10% (vol/vol) 2-β-mercaptoethanol. Nondenaturing PAGE, SDS-PAGE, and silver staining of proteins were performed as described previously (16).

Transmission electron microscopy. The NaCl concentrations in the multicomplex preparation and individual F fractions were equilibrated with HEPES buffer (pH 7) containing 0.1 M NaCl by using a tangential-flow Ultrasette (Filtron Technology Corp., Northborough, Mass.) equipped with a 30,000-M_r-cutoff membrane (Filtron). Cellulase samples (5 μl; 0.02 mg of protein per ml) were placed onto glow-discharged carbon-coated 400-mesh copper grids. The samples were allowed to adsorb to the grid for 5 min before excess was removed by blotting the grid carefully without letting it dry. The samples on grids were washed by placing on them, one at a time, 3 drops of 50 mM NaCl (pH 8.0), allowing each drop to remain for approximately 30 s and then removing excess liquid with filter paper before adding the next drop. This procedure of adding a drop, allowing it to remain for 30 s, and then removing excess liquid was used to stain samples on grids with 3 drops of 2% (wt/vol) aqueous uranyl acetate (unbuffered). When the last drop of staining solution was removed, a thin film of liquid was allowed to remain on the surface. Then, the grids were air dried and

stored in a desiccator. The samples were examined with a Philips CM10 transmission electron microscope operating at 60 kV.

RESULTS

As previously reported (16), the extracellular cellulase system of *C. papyrosolvens* C7 consists of at least seven biochemically distinct multiprotein complexes, each with a different enzymatic and polypeptide composition. These complexes were coeluted from a gel filtration column in a high-molecular-weight fraction (referred to as the multicomplex preparation), and their separation was achieved by subjecting the latter fraction to anion-exchange chromatography (16).

Transmission electron microscopy of the negatively stained multicomplex preparation revealed an array of ultrastructurally diverse particles (5) (Fig. 1A). Some of the particles apparently consisted of loosely aggregated components, whereas other particles were more compact in appearance (Fig. 1A), that is, they comprised components that were tightly packed together.

Anion-exchange chromatography of the multicomplex preparation yielded seven fractions that were designated F1 to F7. Nondenaturing PAGE and SDS-PAGE analysis of individual F fractions, as well as determinations of enzymatic activities, indicated that each F fraction consisted primarily of one biochemically distinct multiprotein complex (16).

In the work described in this paper, we examined the F fractions by transmission electron microscopy to determine whether the structural heterogeneity present in the multicomplex preparation persisted in the individual F fractions or whether individual F fractions consisted primarily of particles of uniform shape. It was thought that, should individual F fractions consist mainly of one morphological type of particle, this finding would be consistent with our previous observation (16) that each F fraction had a distinct enzymatic profile and polypeptide composition.

When the morphologically heterogeneous multicomplex preparation was fractionated by anion-exchange chromatography, much less ultrastructural heterogeneity was observed in negative stains of the individual seven F fractions obtained (Fig. 1B to H) than was observed in the multicomplex preparation. Indeed, most individual F fractions contained, in relatively large numbers, a morphologically distinct type of particle that was present only in smaller numbers, if at all, in other F fractions. For example, a large number of particles in the xylanase-active, Avicelase-inactive fraction F1 consisted of loosely aggregated components clustered in a multiplicity of arrangements (Fig. 1B). Compact particles, abundantly present in the multicomplex preparation, were largely absent from fraction F1. In contrast, the other xylanase-active fraction, fraction F2, primarily contained particles consisting of tightly packed components (Fig. 1C). In fraction F2, we consistently observed particles that appeared to be formed of a ring of globular subunits with conical structures pointing out along the axis on both sides of the ring (Fig. 1C). The diameters of these particles ranged from 14 to 16 nm. We also observed in fraction F2 irregularly shaped particles with diameters similar to those of particles containing a ring of globular subunits. These irregularly shaped particles may represent the same type of particle as the others but in different orientations. Fraction F2 also contained smaller, compact entities with diameters ranging from 3 to 5 nm (Fig. 1C), possibly representing single subunits of the complexes present in the same fraction. Finally, a small percentage of the particles in fraction F2 resembled the loosely constructed particles observed in fraction F1.

In the Avicelase-active fraction F3, triangular or polyhedral particles predominated (Fig. 1D). Low numbers of particles resembling those present in fractions F2 and F4 (see below) were observed in fraction F3. Because fractions F2 and F4 were eluted from the anion-exchange column in proximity to fraction F3 (see Fig. 3 in reference 16), it is probable that some of the particles numerically predominant in fractions F2 and F4 were coeluted in fraction F3. Loose aggregates of particles considerably smaller than fraction F4 particles (see below) were also present in fraction F3.

Fraction F4, the Avicelase-active, major protein fraction that was separated from the multicomplex fraction by anion-exchange chromatography, almost exclusively contained particles consisting of loosely aggregated components (Fig. 1E). These particles were relatively uniform in size but had various shapes, similar to those of particles in fraction F1 (Fig. 1B). However, the subunits of fraction F4 particles had sharper outlines than did the subunits of fraction F1 particles. The component subunits of many of the particles in fraction F4 appeared to be arranged along filamentous structures, which seemed to interconnect the subunits (Fig. 1E and 2). These connections may provide structural flexibility, thus explaining the different arrangements of the component subunits of the particles. No tightly packed particles were observed and only a low level of small particulate debris was detected in fraction F4.

Mixtures of irregularly shaped particles were present in the Avicelase-inactive, CMCase-active fraction F5 (Fig. 1F) and in the Avicelase- and CMCase-active fraction F6 (Fig. 1G). Both fractions contained more or less compact globular particles of different sizes, as well as loosely constructed particles consisting of components with blurred outlines.

Fraction F7, in which only CMCase activity was detected, was rich in particles with tightly aggregated subunits (Fig. 1H). Many of the particles in fraction F7 were star shaped (Fig. 1H) and had diameters of 11 to 15 nm. The relative number of star-shaped particles in this fraction was considerably greater than the number of similar star-shaped particles in the multicomplex preparation. In addition to these particles, other similarly sized but less regularly shaped particles were observed in fraction F7. As mentioned above, some of the irregularities observed in certain structures may be due to viewing identical particles positioned at different angles. Fraction F7 apparently contained more than one morphologically distinct compact particle, inasmuch as a second structural entity observed in this fraction (Fig. 1H) had a diameter (19 nm) larger than that of the star-shaped particles.

In addition to the different types of particles described above, relatively small structures or irregularly shaped fragments were observed in some of the F fractions. These small and/or irregular entities may represent products formed by the dissociation of protein complexes as a result of the negative staining procedure used.

Finally, it should be noted that all the different ultrastructural types of particles observed in fractions F1 to F7 were also seen in negative stains of the multicomplex preparation and, therefore, did not result from the anion-exchange procedure.

DISCUSSION

The results of the ultrastructural analysis reported here support our previous conclusion (16) that the cellulase-xylanase system of *C. papyrosolvens* C7 comprises at least seven different high-molecular-weight multiprotein complexes. In our earlier work the multiprotein complexes were separated by anion-exchange chromatography into seven fractions (F fractions), and it was shown that the multiprotein complexes were bio-

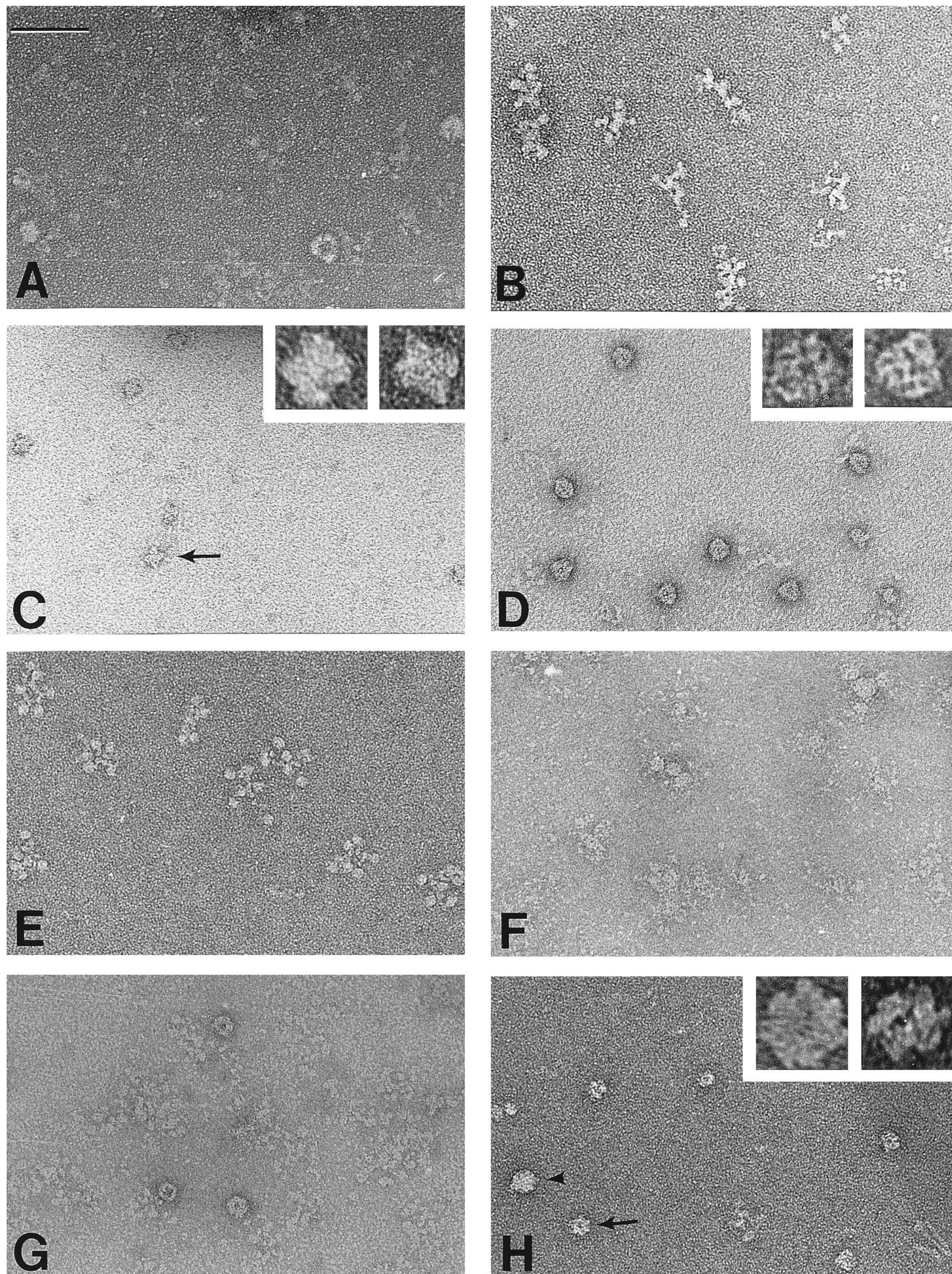

FIG. 1. Transmission electron micrographs of negatively stained F fractions representing the biochemically and structurally distinct cellulase and xylanase multiprotein complexes of *C. papyrosolvens* C7. Shown are the avicelase-active multicomplex fraction separated from culture supernatant fluid by size exclusion chromatography (A) and individual fractions representing the diverse multiprotein complexes (F1 [B], F2 [C], F3 [D], F4 [E], F5 [F], F6 [G], and F7 [H]). Bar, 50 nm. Insets in panels C, D, and H depict 3.2-fold enlargements of the predominant morphological types in F2, F3, and F7, respectively. The arrow in panel C indicates a particle apparently formed of a ring of globular subunits with conical structures pointing out along the axis. The arrow in panel H indicates a star-shaped particle, and the arrowhead indicates a larger, second structural entity.

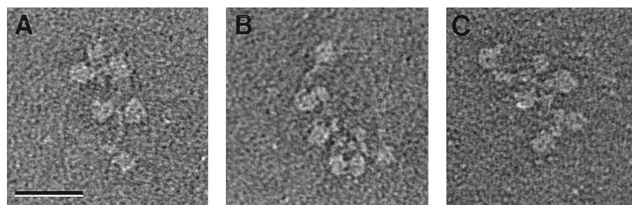

FIG. 2. Transmission electron micrographs of individual loosely aggregated particles in negatively stained fraction F4. The subunits of complexes in this fraction appear to be interconnected by thin filaments. Bar, 20 nm.

chemically distinguishable (16). Analysis of enzymatic activities in culture supernatants and in fractions obtained during the purification procedure indicated that the biochemically distinct complexes were not derived from modifications of what originally was a unicomplex system (16).

In the study reported here we found that most individual F fractions contained, in relatively large numbers, a morphologically recognizable type of particle that occurred only in smaller numbers, if at all, in the other F fractions. Most likely, these morphologically distinct particles represented the biochemically distinct multiprotein complexes that we observed in our previous investigation (16). However, it is possible that the structural flexibility of some of the complexes and/or their different orientations were responsible for some of the morphological diversity observed within the multicomplex preparation and the F fractions.

Various investigators have reported that the components of the cellulase system of *C. thermocellum* and of other anaerobic bacteria are organized as an aggregate or particle, referred to as the cellulosome, which has been shown to be a multiprotein complex consisting of catalytic and noncatalytic polypeptide subunits (2, 6, 7, 11, 14, 18). The catalytic subunits possess endoglucanase, cellobiohydrolase, and hemicellulase activities (14). One of the noncatalytic subunits, named CipA, is a 210- to 250-kDa glycosylated polypeptide that serves as a cellulose-binding factor and a scaffolding polypeptide for the catalytic subunits (2, 8, 12, 14). The cellulase system of *C. thermocellum* has generally been described as a unicomplex system, that is, as consisting of one type of multiprotein complex (the cellulosome). The complex is quite stable, requiring strong denaturing conditions for dissociation (7, 14). A loss of physical integrity of the complex results in greatly diminished efficiency of crystalline cellulose hydrolysis, most likely because the highly ordered arrangement of polypeptides within the complex is required for efficient hydrolysis of the substrate.

Our previous work with the multicomplex cellulase-xylanase system of *C. papyrosolvens* C7 (16) showed that preparations of individual multiprotein complexes had limited ability to hydrolyze crystalline cellulose. However, experiments in which fractions which were eluted from the ion-exchange chromatography column were combined indicated that synergism involving different complexes was required for high rates of crystalline cellulose hydrolysis by the *C. papyrosolvens* cellulase system (16). Apparently, a combination of different multiprotein complexes was necessary for efficient hydrolysis of crystalline cellulose. Thus, it seems likely that different complexes have specific functions in the hydrolysis process.

As pointed out by Béguin and Aubert (2), diversity within cellulase systems may be needed for the hydrolysis of crystalline cellulose because of the physical heterogeneity of the substrate and because the structure of cellulose changes during the degradation process. Furthermore, it may be surmised that efficient hydrolysis of cellulose, as it is present in nature within plant cell walls, requires that cellulase systems retain activity under a multiplicity of environmental conditions and that cellulose be made available to enzymes even though it occurs within a matrix of hemicellulose and other polymers. As mentioned above, it is believed that to achieve maximum rates of cellulose hydrolysis, the polypeptides that make up the cellulase system of anaerobic bacteria must be present in ordered association within complexes. A cellulase system consisting of diverse multiprotein complexes would possess the flexibility needed to efficiently hydrolyze crystalline cellulose under changing environmental conditions and would provide enzymatic activities necessary for extensive degradation of matrix polymers such as xylan.

An earlier report by Mayer et al. (15) described the results of electron microscopy studies of cellulase preparations obtained from cultures of *C. thermocellum* JW20 and YM4. They found that both strains produced a cellulolytic enzyme complex (cellulosome) that, in negative stains, appeared as spherical particles; in strain JW20, such particles were 16 to 18 nm in diameter. These particles consisted of tightly packed subunits and were named "tight cellulosomes." Mayer and coworkers (15) also observed, in cellulosome preparations of strains JW20 and YM4, particles made up of loosely aggregated subunits, which were believed to be derived from the decomposition, or breaking up, of tight cellulosomes. Some of the loose aggregates resembled the particles we observed in fraction F4 in that the subunits of the loose aggregates appeared to be held together by ultrathin fibers (supposedly CipA scaffolding glycopeptides). It is possible that the loose aggregates of *C. thermocellum* subunits were not derived from the tight cellulosomes but constituted a distinct type of multiprotein complex with different polypeptide composition, enzymatic activity, and function(s).

In natural environments, cells of *C. papyrosolvens* C7 may interact with cellulose-containing plant material in a manner different from that described for cells of *C. thermocellum* by Lamed and Bayer (11). For example, inasmuch as *C. papyrosolvens* cells do not possess polycellulosome protuberances on the cell surface and do not adhere to cellulose fibers (4), it may be inferred that they do not form the "contact corridors" reported to connect *C. thermocellum* cells to cellulose fibers (1, 11). Even though growing cells of *C. papyrosolvens* C7 do not attach to their cellulose substrate, they remain close to cellulose fibers (4), probably by means of a chemotactic response similar to that described for other cellulolytic bacteria (9). *C. papyrosolvens* C7 cells release into the external environment as many as seven or more different high-molecular-weight multiprotein complexes which attach to and hydrolyze cellulose and other polysaccharides present in plant material. The extracellular products of polysaccharide hydrolysis (e.g., cellobiose) are utilized as carbon and energy sources by *C. papyrosolvens* and by noncellulolytic facultative anaerobes which serve as O_2 scavengers and secrete vitamins required for the growth of cellulolytic bacteria (3).

ACKNOWLEDGMENTS

We thank C. Woodcock for expert assistance with transmission electron microscopy.

This research was supported by U.S. Department of Energy grant DE-FG02-88ER13898.

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