Serological Study of *Cellulomonas*

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Cell wall antigens from each of six *Cellulomonas* species and two soil isolates were prepared. Cross-reactions were set up with antisera obtained through inoculation of New Zealand white rabbits. The percentage of cross-reaction ranged from 12 to 93. The known cellulomonads clustered in a range of 68 to 93% with one exception: *C. uda* with *C. subalbus* at 43%. The soil isolates demonstrated a much lower affinity and thus were not considered cellulomonads. The cellulomonads, although similar to each other, demonstrated enough diversification to question their reduction to a single species.

The genus *Cellulomonas* has maintained a fluid status ever since Bergey et al. (1) proposed this genus for strains isolated by Kellerman, McBeth, and co-workers (10, 11, 13, 14). To date these organisms have been assigned to three different orders (1-3, 18) and have been recently defined as a single species, *Cellulomonas flavigena* (3).

Cell wall composition of the genus *Cellulomonas* was reported to be uniform (9). However, Cummins (5) cautioned against interpreting qualitative cell wall analyses directly in terms of taxonomic relationships unless they are reinforced by serological and other tests. In view of the similarity of the pattern of cell wall components, it seemed possible that an immunological study of the cell wall from each of six species of *Cellulomonas* and two soil isolates would be useful for studying cross-reactions. Cummins and Harris (4) used cell walls as antigens in agglutination tests, enabling them to identify common antigens in cell walls.

MATERIALS AND METHODS

Cellulomonas species in this investigation were obtained from the American Type Culture Collection. The study included *C. biazotea* ATCC 486, *C. fimi* ATCC 484, *C. flavigena* ATCC 482, *C. gelida* ATCC 488, *C. subalbus* ATCC 489, and *C. uda* ATCC 491. Also included were two soil isolates, JM98A and JM99A, biochemically identified as cellulomonads (19).

Cell wall antigens were prepared from cells serially grown at least three times in TYPG broth, which consisted of the following: tryptone (Difco), 5 g; yeast extract (BBL), 5 g; K₂HPO₄, 5 g; glucose, 0.5 g; and distilled water to 1,000 ml. The pH was adjusted to 7.2. One milliliter of inoculum was transferred to each of five triple-baffled, 2,800-ml Fernbach flasks containing 500 ml of broth and incubated for 24 h at 37 C with agitation at 300 rpm. The cells were harvested by centrifugation at 3,020 × g, at 4 C

for 25 min, washed twice with distilled water, and suspended in distilled water. Disruption of the cells with a French pressure cell (7) was performed at 4 C. Subsequent cell wall treatment was by the method of Robinson (17). Cell walls and insoluble cell debris were harvested by centrifugation at 17,300 × g for 20 min at 4 C. The crude cell wall deposit, resuspended in distilled water, was centrifuged at 480 × g for 10 min at 4 C to remove whole cells and then at 12,100 × g for 15 min to harvest cell walls. The cell wall fractions were sequentially digested with 0.5% (wt/vol) KOH in EtOH; 0.05 M phosphate buffer (pH 7.6) containing 0.5 mg each of ribonuclease and trypsin per ml; and 0.02 M NHCl containing 1 mg of crystalline pepsin per ml. The resulting purified cell wall mucopeptide was washed three times with physiological saline and suspended in 10 ml of saline. All suspensions were stored at 2 C until ready for use.

Protein content of antigen preparations was determined by the method of Folin-Ciocalteu (12) at a wavelength of 740 nm. Standard solutions of crystalline bovine serum albumin (fraction V) were prepared in physiological saline.

Thimerosal was added to the antigen suspension to yield a dilution of 1:10,000. Five New Zealand white rabbits received an antigen preparation 1:1 (antigen:Freund incomplete adjuvant, Difco), and one received an equal amount of saline and Freund incomplete adjuvant. Injections were made subcutaneously on alternate sides for 7 weeks. The total amount of protein injected was as follows: *C. biazotea*, 100 µg; *C. fimi*, 50 µg; *C. flavigena*, 100 µg; *C. gelida*, 100 µg; *C. uda*, 75 µg.

Final bleeding was accomplished by cardiac puncture. Collected sera were filter-sterilized and preserved with Thimerosal at a final dilution of 1:10,000 (8). Antibody titers were determined by the quantitative precipitin and optimal proportions method (8) at the end of 4 and 7 weeks. Using the optimal proportions method, agglutinin titers at the end of 7 weeks were as follows: *C. biazotea*, 1:200; *C. fimi*, 1:50; *C. flavigena*, 1:100; *C. gelida*, 1:100; *C. uda*, 1:200.

Quantitative agglutination tests were performed in triplicate by the method of Kabat and Mayer (8). The homologous and heterologous protein precipitates were quantitated by the Folin-Ciocalteu

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method. The volume of antisera, 0.4 ml, remained constant in all tests.

The percent relatedness was calculated using the following formula: amount of heterologous protein precipitated divided by the amount of homologous protein precipitated $\times 100$. The protein values were corrected for the amount of antigen added and for the amount of precipitate formed in the saline control for the homologous system. Optimum precipitation in the homologous system was obtained by adding 0.06 ml of antigen solution. Therefore, this amount was added in all heterologous tests.

RESULTS

The percent cross-reaction varying with species ranged from 43 to 93. The soil isolates, initially classified as *Cellulomonas* species, demonstrated a much lower affinity than the type strains, with a maximum cross-reaction of 56% and a low of 12% (Fig. 1 and 2).

DISCUSSION

The quantitative agglutination procedure indicated that the cellulomonads had common

cell wall antigens. However, in no instance did a heterologous reaction equal the homologous. Thus, the reduction of these organisms to a single species was questionable. The weaker reactions of *C. gelida* and *C. uda* with the antisera from the other cellulomonads suggested that the two have fewer common cell wall antigens. This was supported to some extent by Keddie et al. (9). Their work differentiated *C. uda* by the presence of glycine as a major cell wall component. The two strongest relationships were exhibited by *C. biazotea* with *C. flavigena* and *C. gelida* with *C. uda*. However, in a 70-character, single-linkage computer analysis, Davis and Newton (6) presented a dendrogram where *C. flavigena* and *C. biazotea* achieved a 77% similarity. Since these were the only two cellulomonads tested, it was not possible to make a comparison with the other species. In other computer analyses of coryneform bacteria, Masuo and Nakagawa (15, 16) showed five species of *Cellulomonas* to

FIG. 1. Serological relation of *Cellulomonas* species. The results are the averages of reciprocal cross-reactions. Six different determinations were made.

FIG. 2. Serological relation of *Cellulomonas* species to *C. subalbus* and unknown soil isolates. The results are the averages of three determinations.

be 80 to 90% similar based on 84 characters in one instance and 106 in the other. The serological data presented here indicated a much greater differentiation. The range of cross-reaction varied from a low with *C. uda* and *C. subalbus* of 43% to a high of 93% for *C. flavigena* with *C. biazotea* and *C. uda* with *C. gelida*.

Although the cellulolytic soil isolates resembled the cellulomonads morphologically and biochemically, they demonstrated a much lower affinity for the cell wall antigens. The results indicated that JM98A had fewer common cell wall antigens than JM99A, but neither approached the magnitude of the cross-reactions of the known *Cellulomonas* species with one exception. *C. uda* seemed to share

more cell wall antigens with the soil isolate JM99A than with *C. subalbus*. Thus, it seemed from this study that, although the *Cellulomonas* species shared many of their cell wall antigens, the strains were not identical. Reduction of these organisms to a single species should be the subject of further investigation.

ACKNOWLEDGMENTS

This investigation was supported in part by funds provided by the International Center for Arid and Semi-Arid Land Studies, Texas Tech University, and the National Science Foundation NSF GR #GU-3759.

REPRINT REQUESTS

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