Immunological Study of Pullulanase from *Klebsiella* Strains and the Occurrence of this Enzyme in the *Enterobacteriaceae*

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The abilities of various *Enterobacteriaceae* strains to grow on starch or pullulan and the occurrence of pullulanase in these strains were studied. Only the *Klebsiella* strains grew well on amylopectin and pullulan and produced pullulanase. None of the *Enterobacter* strains produced pullulanase. The pullulanase produced by various strains of *Klebsiella* was examined by the immunodiffusion method. Culture filtrates and cell extracts of all of the *Klebsiella* strains tested formed a precipitin line against antiserum prepared with crystalline pullulanase from *K. pneumoniae* IFO 3321. Except for *K. pneumoniae* IFO 3317 and ATCC 21073, these lines fused completely with the homologous line. No materials which cross-reacted with the antiserum were produced by the other *Enterobacteriaceae* strains studied. The production of the enzyme is discussed as a character of potential use in the classification and identification of strains of *Enterobacteriaceae*.

Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase) is an enzyme that hydrolyzes the (1→6)- α -glucosidic linkages in pullulan to form maltotriose as the final product. Pullulanase was discovered by Bender and Wallenfels (3) in *Aerobacter aerogenes*, strains of which are now classified as members of either *Enterobacter* or *Klebsiella*. The enzyme has been reported to be formed in one or more strains of *Escherichia intermedia* (23), *Streptococcus mitis* (24), *Bacillus cereus* subsp. *mycoides* (22), and *Lactobacillus plantarum* (28). The pullulanase-producing strain of *E. intermedia* (ATCC 21073) has been reported by the American Type Culture Collection (ATCC) (2) to be an aberrant strain of *Klebsiella pneumoniae*.

Pullulanase, together with isoamylase, which was discovered by Harada et al. (10) in *Pseudomonas amyloclavata*, has been used to elucidate the structures of polysaccharides and to produce useful materials, such as maltose and amylose, by debranching starch with and without β -amylase. We have compared the actions (9, 27) and molecular properties (1, 12) of the two enzymes previously.

Previously (26) we showed that *Escherichia coli*, *Salmonella typhimurium*, and *Citrobacter freundii* produce materials without enzyme activity that cross-react with anti-*K. aerogenes* arylsulfatase serum under conditions in which *klebsiellae* produce arylsulfatase. Thus, we became interested in the distribution of pullulanase and of the enzymically inactive protein in strains of the family *Enterobacteriaceae*. In this work, antiserum against *K. pneumoniae* pullu-

lanase was prepared, and the distribution of pullulanase among some *Enterobacteriaceae* strains was studied enzymatically and immunologically.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12(λ), *S. typhimurium* LT2, and *K. aerogenes* W70 were obtained from H. Ogawa, Osaka University, T. Fukazawa, Keio University, and S. Yamagishi, Chiba University, respectively. *K. pneumoniae* ATCC 21073, previously identified as a strain of *E. intermedia* by Ueda and Nanri (23), who isolated the strain, was obtained from the ATCC. All other strains used were obtained from the Institute for Fermentation (IFO), Osaka.

Culture conditions. The medium used (pH 7.2, 100 ml) was composed of: $(\text{NH}_4)_2\text{HPO}_4$, 0.15 g; KH_2PO_4 , 0.1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g; NaCl , 1 mg; CaCl_2 , 1 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1 mg; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5 μg ; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2 μg ; $\text{ZnCl}_2 \cdot 7\text{H}_2\text{O}$, 1 μg ; H_3BO_3 , 1 μg ; and a carbon source, 1 g. Unless otherwise stated, organisms were grown aerobically at 30°C for 4 days in 500-ml conical flasks by inoculating 5 ml of seed culture in this medium into 95 ml of fresh medium.

For the formation of pullulanase, organisms were grown in the same medium supplemented with 0.5% sodium glutamate, since this compound enhances formation of pullulanase. Cells were harvested by centrifugation at 10,000 $\times g$ for 30 min. The supernatant was assayed for pullulanase activity and immunological reactivity, whereas the precipitated cells were washed twice with saline and once with sodium phosphate buffer (pH 7.0) and stored at -20°C until used.

Preparation of cell extracts. Suspensions of cells in 0.02 M sodium phosphate buffer (pH 7.0) were treated with 0.1% (final concentration) sodium dodecyl sulfate for 20 h at 30°C by the method of Ohba and

Ueda (15). The mixtures were centrifuged, and the resultant supernatants were dialyzed against the buffer described above and used for enzymatic and immunological assays. Cell extracts obtained by sonic oscillation for 20 min at 20 kcycles and at $<6^{\circ}\text{C}$ were used as controls.

Enzyme assay. Pullulanase activity was measured in 2 ml of reaction mixture consisting of 1 ml of 1% pullulan solution in 0.1 M acetate buffer (pH 5.5) and the enzyme solution. The reaction was carried out for 15 min at 40°C , and the release of aldehyde groups was then determined by the Nelson method (13). One unit of activity was defined as the amount of enzyme causing liberation of 1 μmol of aldehyde group per min.

Preparation of antiserum. Three 6-month-old male New Zealand white rabbits were injected intramuscularly with 2 ml of an emulsion consisting of 1 ml of Freund incomplete adjuvant, 1 ml of 0.85% NaCl in 0.02 M sodium phosphate buffer (pH 7.4), and 1 mg of pure pullulanase from *K. pneumoniae* IFO 3321. As booster injections, they were given the same type of injection 2 weeks later and then subcutaneous injections of 1 mg of the enzyme in incomplete adjuvant at 2-week intervals. Blood was collected from an ear vein 7 days after each booster injection. Control sera were obtained from rabbits before immunization.

Immunodiffusion. Experiments were carried out by the Stollar and Levine modification (21) of the Ouchterlony technique. Glass plates (2.6 by 7.6 cm) were coated with 1.1% agar in sodium phosphate buffer-saline (pH 7.4) to a thickness of 0.1 cm. Two microliters of undiluted antiserum followed by 2- μl volumes of concentrated culture supernatant and cell extracts or appropriately diluted samples of pullulanase were put into the sample wells. Sufficient material was added to sample wells to give 50 to 100 mU of pullulanase activity if enzyme activity was present, or three times as much protein as that of the preparation from *K. pneumoniae* IFO 3321 if activity was not present. Samples were concentrated by precipitation with ammonium sulfate. Plates were incubated overnight in a high-humidity chamber at 30°C and then examined for precipitin lines. The plates were washed eight times with 0.85% NaCl to remove non-precipitated protein and were then stained with 0.5% amido black.

Immuno-electrophoresis. The homogeneity of pullulanase antiserum was tested immuno-electrophoretically by a minor modification of the Scheidegger method (19). Wells 2 mm in diameter were punched in a coating of 1% agarose (in barbital-acetate buffer, pH 8.6) on a glass slide (5 by 8 cm). Approximately 2 μl of crude or purified enzyme solution was placed in each well. After electrophoresis was carried out at 5 to 7 V/cm for 60 min with the same buffer, 140 μl of pullulanase antiserum was put into the trough, and the slide was placed overnight in a high-humidity chamber at 30°C . The slide was then washed with 0.85% NaCl to remove excess protein and was stained with 0.5% amido black.

Chemicals. Crystalline pullulanase and pullulan were kindly supplied by Hayashibara Biochemical Laboratory, Okayama, Japan. Waxy corn amylopectin was obtained from Nichiden Kagaku Industries, Osaka, Japan. Soluble starch was purchased from

Wako Pure Chemicals, Osaka, Japan. Other chemicals were commercial products of the highest purity available.

RESULTS

Specificity of antipullulanase. When homologous crystalline pullulanase from *K. pneumoniae* IFO 3321 and the supernatant of a culture of the same strain grown with soluble starch were tested by the immunodiffusion method, both preparations gave a single sharp precipitin line, and these lines fused completely, indicating that the two antigens were identical and also that the antiserum was immunologically pure (Fig. 1). These findings were confirmed by immuno-electrophoresis of crystalline and crude preparations of the enzyme (Fig. 2). The slight difference in mobility of the two samples was probably due to the effect of impurities in the crude preparation. Serum from nonimmunized animals did not show any reaction with the pullulanase preparations.

Pullulanase was shown by Wallenfels et al. (25) and Fujio et al. (8) to be formed either extracellularly or in a cell-bound state, depending upon the culture conditions. The lines

FIG. 1. Immunodiffusion of pullulanase from *K. pneumoniae* IFO 3321 with antiserum. Purified and crude enzyme preparations (2 μl ; 50 mU) were placed in wells 1 and 2, respectively, and antiserum (2 μl) was placed in the upper well.

FIG. 2. Immuno-electrophoresis of pullulanase from *K. pneumoniae* IFO 3321 with antiserum. Purified and crude enzyme preparations (2 μl ; 140 mU) were placed in wells 1 and 2, respectively, and antiserum (140 μl) was placed in the center trough.

formed by the preparations of extracellular and intracellular enzymes fused completely with each other. Thus, both enzymes were found to be the same immunologically.

Growth of various strains of *Enterobacteriaceae* on amylopectin, soluble starch, or pullulan, and pullulanase formation by the strains. The occurrence of pullulanase in strains of *Enterobacteriaceae* was examined. A debranching enzyme such as pullulanase is a com-

ponent of the starch- or pullulan-utilizing system in several bacteria. We first investigated utilization, by the strains, of amylopectin, soluble starch, and pullulan as sole carbon sources.

All eight strains of *Klebsiella* grew well on waxy corn amylopectin, soluble starch, and pullulan (Table 1). None of the other strains tested grew appreciably on waxy corn amylopectin, but *E. coli* K-12(λ), *S. typhimurium* LT2, all strains of *Enterobacter* tested, and *Serratia marces-*

TABLE 1. Growth of various strains of *Enterobacteriaceae* on waxy corn amylopectin, soluble starch, and pullulan, and pullulanase activity in the supernatants of maltose-grown cultures

Strain ^a	Growth (A_{600}) ^b			Pullulanase formation in maltose medium		
	Waxy corn amylopectin	Soluble starch	Pullulan	Growth (A_{600}) ^b	Activity ^c (U/ml)	
<i>Escherichia coli</i> K-12(λ)	0.0	1.1	0.0	4.8	<0.001	(<0.01)
<i>E. coli</i> B	0.0	0.0	0.0	5.1	<0.001	
<i>Citrobacter freundii</i> IFO 12681	0.0	0.0	0.0	6.3	<0.001	
<i>Salmonella typhimurium</i> LT2	0.0	0.8	0.0	4.4	<0.001	(<0.01)
<i>Klebsiella aerogenes</i> W70	1.1	2.8	3.0	6.3	0.18	(1.5)
<i>K. pneumoniae</i> IFO 3317	1.6	2.6	2.6	6.0	0.004	(0.05)
<i>K. pneumoniae</i> IFO 3318	1.5	2.6	2.5	6.2	0.15	(1.1)
<i>K. pneumoniae</i> IFO 3319	1.5	2.5	2.7	4.4	0.22	(1.7)
<i>K. pneumoniae</i> IFO 3321	1.2	1.6	2.2	3.9	0.20	(1.6)
<i>K. pneumoniae</i> IFO 12059	1.4	2.6	1.8	7.9	0.01	(0.10)
<i>K. pneumoniae</i> IFO 13541	1.1	2.5	2.0	5.9	0.05	(0.24)
<i>K. pneumoniae</i> ATCC 21073 (<i>Escherichia intermedia</i>)	1.4	2.8	0.8	6.0	0.04	(0.32)
<i>Enterobacter cloacae</i> IFO 3320	0.0	0.6	0.0	3.0	<0.001	(<0.01)
<i>E. cloacae</i> IFO 12935	0.0	1.1	0.0	6.7	<0.001	(<0.01)
<i>E. cloacae</i> IFO 12937	0.0	1.7	0.0	7.4	<0.001	(<0.01)
<i>E. cloacae</i> IFO 13535	0.0	1.7	0.0	6.4	<0.001	(<0.01)
<i>E. cloacae</i> IFO 13536	0.0	0.5	0.0	5.6	<0.001	(<0.01)
<i>E. aerogenes</i> IFO 12010	0.0	0.7	0.2	6.2	<0.001	(<0.01)
<i>E. aerogenes</i> IFO 13534	0.0	0.6	0.2	6.7	<0.001	(<0.01)
<i>E. liquefaciens</i> IFO 12979	0.0	1.6	0.0	8.0	<0.001	(<0.01)
<i>E. liquefaciens</i> IFO 13537	0.0	0.9	0.1	5.0	<0.001	(<0.01)
<i>Serratia marcescens</i> IFO 3046	0.0	0.0	0.2	1.8	<0.001	
<i>S. marcescens</i> IFO 3735	0.0	1.1	0.2	4.4	<0.001	(<0.01)
<i>S. marcescens</i> IFO 12648	0.0	1.2	0.2	3.9	<0.001	(<0.01)
<i>S. marcescens</i> IFO 12946	0.0	0.5	0.2	3.9	<0.001	(<0.01)
<i>Proteus inconstans</i> IFO 12930	0.0	0.0	0.0	3.0 ^d	<0.001 ^d	
<i>P. morganii</i> IFO 3168	0.0 ^e	0.0 ^e	0.1 ^e	0.7 ^d	<0.001 ^d	
<i>P. mirabilis</i> IFO 3849	0.1 ^e	0.0 ^e	0.0 ^e	3.3 ^d	<0.001 ^d	
<i>P. vulgaris</i> IFO 3045	0.1 ^e	0.2 ^e	0.2 ^e	4.5 ^d	<0.001 ^d	
<i>P. vulgaris</i> IFO 3167	0.1 ^e	0.2 ^e	0.3 ^e	8.1 ^d	<0.001 ^d	
<i>P. vulgaris</i> IFO 3851	0.0 ^e	0.1 ^e	0.2 ^e	6.8 ^d	<0.001 ^d	
<i>P. vulgaris</i> IFO 3988	0.1 ^e	0.1 ^e	0.2 ^e	2.3 ^d	<0.001 ^d	
<i>P. rettgeri</i> IFO 13501	0.0	0.0	0.0	2.5 ^d	<0.001 ^d	
<i>Erwinia amylovora</i> IFO 12687	0.0 ^e	0.0 ^e	0.1 ^e	1.8 ^d	<0.001 ^d	
<i>E. carotovora</i> IFO 3380	0.0	0.0	0.2	1.2 ^d	<0.001 ^d	

^a The organisms were grown aerobically for 4 days at 30°C (or 27°C for *E. amylovora* IFO 12687) in the synthetic medium as described in the text but containing 1% of the sugar indicated.

^b Numbers indicate increases in absorbance at 600 nm.

^c Numbers in parentheses represent pullulanase activities in soluble starch medium.

^d Yeast extract (0.1% final concentration) was added to the synthetic medium because these strains were unable to grow well in the medium.

^e Yeast extract and peptone (0.1% each final concentration) were added to the synthetic medium because these strains were unable to grow in the medium. The numbers indicate values with sugar minus those without sugars.

cens IFO 3735, IFO 12648, and IFO 12946 grew fairly well on soluble starch. All of the *Enterobacter aerogenes* and *S. marcescens* strains tested, some *Proteus* strains, and *Erwinia carotovora* IFO 3380 grew slightly on pullulan; these strains seemed to produce trace amounts of pullulanase or of some other pullulan-degrading enzymes. *E. coli* K-12(λ), *S. typhimurium* LT2, all strains of *Enterobacter cloacae* tested, and *Enterobacter liquefaciens* IFO 12979, which can grow on starch but not on pullulan, seemed to produce another amylase.

Pullulanase activity (Table 1) was assayed in the supernatants of cultures grown in maltose medium, which induces pullulanase in *K. pneumoniae* (7); the soluble-starch medium was also used for assays of strains which grew in this medium. Under the culture conditions used, pullulanase was formed mainly in the supernatant. All eight strains of *Klebsiella* studied produced pullulanase; none of the other strains tested produced any detectable pullulanase in either medium, even when the supernatant was concentrated 20 times by salting-out and incubated with substrate for 24 h. Moreover, none of the cell extracts obtained by sonic oscillation or sodium dodecyl sulfate extraction except those of *Klebsiella* strains showed any activity. The culture supernatants of *E. coli* K-12(λ), *Enterobacter cloacae* IFO 12937, IFO 13535, and IFO 13536, *Enterobacter aerogenes* IFO 12010 and IFO 13534, *Enterobacter liquefaciens* IFO 12979, and *S. marcescens* IFO 3735 and IFO 12648 contained activity for decomposition of soluble starch, which activity was detected by measuring the increase of reducing sugar.

Pullulanase has been known to be produced by many strains of *Aerobacter aerogenes* (3, 5, 7, 9), which are now classified as members of *Enterobacter* or of *Klebsiella*. However, no pullulanase has been detected among strains of *Enterobacter*.

Immunodiffusion of pullulanase from *Klebsiella*. The eight strains of *Klebsiella*, which produce pullulanase, were cultured in soluble-starch medium, and the supernatants were tested for reactivity against antiserum from *K. pneumoniae* IFO 3321 pullulanase. The culture supernatants from all of the strains cross-reacted with the antiserum (Fig. 3A and B). The precipitin lines of the preparations from *K. aerogenes* W70 and *K. pneumoniae* IFO 3318, IFO 3319, IFO 3321, IFO 12059, and IFO 13541 fused completely with each other (Fig. 3A). Those of the preparations from *K. pneumoniae* IFO 3317 and *K. pneumoniae* ATCC 21073 also fused completely with each other; however, they formed a spur with the homologous line (Fig. 3B), an indication that these pullulanases do not possess

FIG. 3. Immunodiffusion experiment demonstrating cross-reactions between pullulanases of *Klebsiella* and antiserum to pullulanase from *K. pneumoniae* IFO 3321. The center wells contained 2 μ l of antiserum. (A) Sample wells contained concentrated culture supernatants (2 μ l; 50 to 100 mU) from the following strains: (1) IFO 3321; (2) W70; (3) IFO 3318; (4) IFO 3319; (5) IFO 12935; and (6) IFO 13541. (B) Sample wells contained concentrated culture supernatants (2 μ l; 50 to 100 mU) from the following strains: (1,4) IFO 3321; (2,5) IFO 3317; and (3,6) ATCC 21073.

all of the antigenic determinants found in the pullulanase of the other strains. The extracts of the cells obtained by treatment with sodium dodecyl sulfate gave similar reactions.

To determine whether any proteins without pullulanase activity but with cross-reactivity against antipullulanase were produced, concentrates of culture supernatants and cell extracts from other strains of *Enterobacteriaceae* were tested by the diffusion method. However, no such proteins were detected in the preparations.

DISCUSSION

Enevoldsen et al. (6) reported a new method, using antibodies towards crude pullulanase for cross-immunoelectrophoresis, to follow the pu-

rification of this enzyme, but they did not report the immunological properties of the antibody towards pullulanase. Our immunological analysis showed that the pullulanases of *Klebsiella* can be divided into two groups: the first contains the pullulanases of *K. aerogenes* W70 and *K. pneumoniae* IFO 3318, IFO 3319, IFO 3321, IFO 12059, and IFO 13541, and the other contains the pullulanases of *K. pneumoniae* IFO 3317 and ATCC 21073. The former are homologous, but the latter are heterologous, because their precipitin lines form a spur with the homologous line. Previously we found that the precipitin line of strain 3317 with arylsulfatase fused partially with the homologous line to the antiserum prepared against pure arylsulfatase of *K. aerogenes* W70, whereas the lines of other arylsulfatase-positive strains of *K. pneumoniae* fused completely (26). These immunological results with the two enzymes suggest that strain 3317 differs significantly from other strains of *K. pneumoniae*. Another strain, ATCC 21073, which produces heterologous pullulanase, was originally isolated by Ueda and Nanri (23) and identified as *Escherichia intermedia*. Strains of *E. intermedia* are now classified as members of *Citrobacter intermedius* (20); however, the above-mentioned isolate is classified as an aberrant strain of *K. pneumoniae* (2). This strain differs from other *K. pneumoniae* strains with respect to the Voges-Proskauer and methyl red reactions, and, as reported here, its pullulanase is immunologically different from those of other *K. pneumoniae* strains. Hori and Osawa (11) conducted a taxonomic study involving ribosomal protein composition and reported that strains IFO 3317 and ATCC 21073 differ from other *Klebsiella* strains and belong to one and the same group.

Among the strains of *Enterobacteriaceae* tested, *E. coli* K-12(λ), all strains of *Enterobacter*, and *S. marcescens* IFO 3735, IFO 12648, and IFO 12946 grew well on soluble starch but did not produce pullulanase. Only strains of *Klebsiella* produced pullulanase activity and material with immunological reactivity against anti-pullulanase. In *Bergey's Manual of Determinative Bacteriology*, 8th ed. (16, 18), all motile *Aerobacter* strains were placed in *Enterobacter*, and the nonmotile strains were placed in *Klebsiella*. This classification is consistent with that based on pullulanase formation. Thus, pullulanase formation may be a character common to all klebsiellae and, if so, it may be a key character in differentiating strains of *Klebsiella* from other strains of *Enterobacteriaceae*. Obviously, to determine this, large numbers of strains will have to be studied.

Palmer et al. (17) reported the occurrence of

a pullulanase in *E. coli* ML, but Dessein and Schwartz (3) were dubious about this finding, since on careful examination they themselves detected no pullulanase in 11 strains of *E. coli*, including strain ML. We could not detect pullulanase enzymatically or immunologically in strains K-12(λ) and B of *E. coli*.

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REPRINT REQUESTS

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