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Chitinase-Overproducing Mutant of *Serratia marcescens*

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Genetic modification of *Serratia marcescens* QMB1466 was undertaken to isolate mutants which produce increased levels of chitinolytic activity. After mutagenesis with ultraviolet light, ethyl methane sulfonate or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 19,940 colonies were screened for production of enlarged zones of clearing (indicative of chitinase activity) on chitin-containing agar plates. Forty-four chitinase high producers were tested further in shake flask cultures. Mutant IMR-1E1 was isolated which, depending on medium composition, produced two to three times more endochitinase activity than the wild type. IMR-1E1 also produced two to three times more than the wild type of the other components of the chitinolytic enzyme system—a factor involved in the hydrolysis of crystalline chitin and chitobiase. After induction by chitin, endochitinase and chitobiase activity appeared at similar times for both IMR-1E1 and QMB1466, suggesting possible coordinate control of these enzymes. The results are consistent with IMR-1E1 containing a regulatory mutation which increased production of the components of the chitinolytic enzyme system and/or with IMR-1E1 containing a tandem duplication of the chitinase genes. The high rate of reversion of IMR-1E1 to decreased levels of chitinase production suggests that the overproduction of chitinase by IMR-1E1 is due to a tandem gene duplication.

Chitin is an unbranched polysaccharide composed primarily of β-1,4 linked *N*-acetylglucosamine (NAG) residues with occasional glucosamine residues (3). It can be regarded as a cellulose analog in which the C_2 hydroxyl groups have been replaced by *N*-acetylamido groups. Chitin is the principal structural component of insect exoskeletons and of crustacea such as shrimp and crab (3). The processing of these shellfish generates a waste disposal problem, and chitinase is essential to a bioconversion process in which waste shellfish chitin is enzymatically hydrolyzed to NAG which in turn is assimilated by an edible yeast (4).

A microbial source of chitinase was sought, and *Serratia marcescens* QMB1466 was chosen since it was one of the most active chitinase producers found of the 400 chitinolytic fungi and bacteria tested (4, 11). *S. marcescens* was also chosen because its chitinase has some activity against "crystalline" chitin (11) and because it is related to *Escherichia coli*. Potentially, many of the advanced genetic manipulations possible with *E. coli* might be adapted to *S. marcescens*.

The chitinase system of *S. marcescens* resembles that of most chitinolytic eucaryotes and procaryotes (8, 12) in that chitin is hydrolyzed to NAG by two separate hydrolases: (i) an endochitinase [poly-β-1,4-(2-acetamido-2-deoxy)-D-glucoside glycanhydrolase (EC 3.2.1.14)], which produces low-molecular-weight, soluble multimers of NAG, the dimer *N,N'*-diacetyl chitobiose being predominant, and (ii) a chitobiase (chitobiose acetylamidodeoxyglucohydrolase [EC 3.2.1.29]), which hydrolyzes the intermediates to NAG. The *S. marcescens* chitinase system differs from many other chitinolytic systems which act only on swollen chitin in that it hydrolyzes "crystalline" chitin.

In this paper we describe the isolation of a chitinase-overproducing strain of *S. marcescens* QMB1466 isolated by several cycles of mutation and selection. Results on "crystalline" chitin hydrolysis and chitobiase production by the overproducing mutant are presented, and the genetic basis for the overproducing phenotype is discussed.

MATERIALS AND METHODS

Bacteria. *S. marcescens* QMB1466 was obtained from E. T. Reese from the U.S. Army Natick Laboratory Culture Collection (11).

Chitin preparation. A variation of the Monreal and Reese (11) procedure was used to prepare swollen chitin. Twenty-five grams of chitin (Calbiochem) was ball milled, and the fraction recovered between 100- and 200-mesh screens was suspended in 250 ml of 85% H_3PO_4 and stored at 4°C for 24 h. This mixture was suspended in 2 liters of deionized water using a Waring blender and washed until the pH was 5.0 to 5.5. Sodium hydroxide (1 N) was then added to raise the pH to 7.0. This mixture was centrifuged (8,000 × *g*, 10 min), suspended in 1 liter of deionized water, and centrifuged (8,000 × *g*, 10 min), and the pelleted chitin was stored at 4°C. The pellet contained from 7 to 10%

(wt/wt) of swollen chitin. Chitin concentration was calculated by drying to constant weight (approximately 4 days at 53°C).

Media. M9C medium (pH 6.9) was composed of M9 minimal medium (10) with 10 mg of swollen chitin per ml substituted for glucose. One microgram of thiamine per ml was added after salts and substrate (autoclaved separately) were cooled and combined. Reese medium (pH 7.7) consisted of salts, 15 mg of swollen chitin per ml, and yeast extract and buffer (11). LB medium (10) contains tryptone, yeast extract, and sodium chloride.

LB and M9C agar plates contained 15 mg of agar per ml. NAC plates contained 10 mg of nutrient agar (Difco) per ml, 10 mg of agar per ml and 10 mg of swollen chitin per ml. Chitinase assay agar contained 10 mg of swollen chitin per ml as substrate, 15 mg of agar per ml, 625 μg of sodium azide per ml in 0.05 M citrate-phosphate buffer (pH 6.6).

Chitinase preparation. Shake flask culture supernatants containing chitinase were obtained by centrifugation (5,900 × *g*, 15 min, 4°C). The approximate half-life of the QMB1466 chitinase at pH 7.0 at 4°C was 2 months.

Chitinase assays. The chitinolytic activity assay of Monreal and Reese (11) involving shaking swollen chitin at 50°C for 1 h in the presence of enzyme was used. Enzyme boiled for 5 min was used in controls. At the beginning of this study reducing sugar concentration in the hydrolysate was determined by the dinitrosalicyclic acid (DNS) (Eastman Kodak) method (9, 16) or the Nelson-Somogyi copper reduction method (15) using NAG (U.S. Biochem) as a standard. Later in the study, NAG concentration in hydrolysates was measured using *p*-dimethylaminobenzaldehyde (DMAB) (Aldrich) reagent (13). One unit of activity equals 1 μmol of reducing sugar released per min. The Nelson-Somogyi method measures reducing sugars whereas the DMAB assay is specific for NAG. The value of chitinase activity determined by the Nelson-Somogyi method was 30% higher than that determined by the DMAB assay. Results of chitinase activity assays obtained using DMAB were multiplied by 1.3 for calculations of chitinase units.

Chitinase diffusion assays were done by depositing 40-μl volumes of supernatant in 6-mm-diameter wells made in chitinase assay agar plates (15 ml of agar per plate). Zones of clearing (indicative of chitinase activity) which formed around the wells were measured after 72 h at 42°C. The logarithm of chitinase concentration plotted against the diameter of the zone of clearing resulted in a straight line (Fig. 1).

Chitobiase assay. A spectrophotometric method for measuring chitobiase activity based on hydrolysis of *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (NPGlu) was developed (17). The substrate (980 μl of 2 mM NPGlu [Sigma] in 0.02 M citrate-NaOH [pH 7.5]) was incubated at 37°C; 40 μl of appropriately diluted enzyme was added, and the reaction was quenched after 15 min by adding 980 μl of 0.5 M glycine-NaOH (pH 11.0). Absorbance was measured at 400 nm, and the blank was prepared by incubating enzyme with the quenching solution. One unit of activity equals 1 μmol of *p*-nitrophenol released per min.

Chitobiase activity was also measured by determining the amount of NAG produced from chitobiose in the presence of enzyme. The substrate (0.5 ml of 2 mM *N,N'*-diacetyl chitobiose [Sigma] in 0.02 M citrate-NaOH [pH 7.5]) was placed in a water bath at 50°C, 10 μl of appropriately diluted enzyme was added, and the reaction was stopped after 60 min by boiling for 1 min. The amount of NAG produced was measured by the DMAB assay and corrected with a blank containing boiled enzyme. One unit of activity equals 1 μmol of NAG released per min.

Crystalline chitin hydrolysis. Crystalline chitin hydrolysis was measured by the Monreal and Reese assay (CH_1 assay) using milled chitin as substrate (11). The amount of NAG released was determined with the DMAB reagent, and one unit of activity equals 1 μmol of NAG released per min.

Mutagenesis. Survival curves were constructed for each mutagen using either the wild-type organism or the appropriate mutant. The level of mutagenesis found to give the highest ratio of auxotrophs to survivors was used in further experiments. Mutagenesis by ultraviolet light (10), ethyl methane sulfonate (Eastman Kodak) (10), and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich) (10) was followed by four to five generations of growth in LB to allow phenotypic expression. Survivors were plated on NAC medium and scored after 36 h of incubation at 30°C.

Mutant isolation and characterization. (i) Plate screen. Colonies which formed larger zones of clearing than the wild type on NAC plates at 30°C were purified and stabbed onto fresh NAC plates. Clones showing the largest zones of clearing after 36 h were screened in shake flasks.

(ii) Shake flask screening. Duplicate 125-ml baffled flasks (Bellco) containing 25 ml of M9C medium

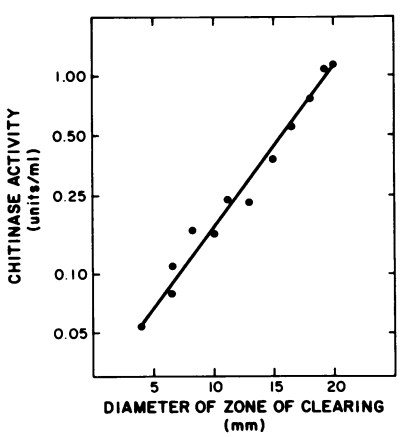

FIG. 1. *Estimation of chitinase activity using the agar diffusion assay. A 40-μl volume of solutions of known chitinase activity (DNS assay) was pipetted into wells in chitin agar plates. Concentrated chitinase was obtained by ultrafiltering supernatants (Diaflo UM 10 membrane, Amicon). The plates were incubated at 42°C for 72 h, and the size of the zones of clearing was measured. The diameter of the well (6 mm) was subtracted from the diameter of the zone clearing.*

were inoculated with 0.1 ml of culture grown overnight in LB. In some experiments 5 mg of swollen chitin per ml was added to LB medium to preinduce the culture. Changes in pH, cell number (microscope counts), and chitinase activity of the supernatant were measured over 5 to 6 days. Incubation was at 30°C at 130 rpm. Antifoam solution (25 or 50 μl) consisting of polypropylene glycol 2000 (Polysciences) 1:10 in ethanol was added to shake flasks after 24 to 36 h.

(iii) Chitinase production. For purposes of comparison with results reported by Monreal and Reese (11), maximum chitinase production was measured in shake flasks containing Reese medium. Cultures were prepared as previously described for shake flask screening. In certain experiments yeast extract was omitted from Reese medium.

RESULTS

Chitinase activity of *S. marcescens* QMB1466. The maximum chitinase activity we obtained for *S. marcescens* QMB1466 grown in Reese medium (with yeast extract) and assayed using DNS was 0.292 U per ml of supernatant (SD = 0.033, $n = 5$; SD is the standard deviation and n is the number of cultures assayed). This value is similar to the 0.283 U/ml reported by Monreal and Reese (10) for QMB1466 grown and assayed under the same conditions. This value (0.283 U/ml) is 68 chitinase units per ml in the notation used by Monreal and Reese (11) which is derived from the cellulose filter paper assay for cellulase (6).

The DNS assay was not used in later experiments because of non-linearity and lack of sensitivity at low NAG concentrations and because of interference caused by citrate in the enzyme assay buffers (7; M. Young, personal communication).

Isolation of chitinase-overproducing mutant. Over 14,000 survivors of ultraviolet mutagenesis were screened on NAC plates for chitinase production (Table 1). Dose levels between 50 and 3,000 ergs/mm^2 were used, and percentage survival ranged from 0.003 to 3%. Over 600 colonies which formed the largest zones of clearing on NAC plates were retested on NAC plates, and 39 clones showing the largest zones of clearing were screened for chitinase production in shake flasks in M9C medium.

For the shake flask screening, yeast extract was not used in either the Reese or M9C media so that chitin would be the sole carbon source and to help alleviate the foaming problem. In the absence of yeast extract, chitinase production was lower in M9C medium (0.208 U/ml, SD = 0.054 U/ml, $n = 6$) than in Reese medium (0.246 U/ml, SD = 0.096 U/ml, $n = 8$). M9C medium was used for screening, because in M9C variation in chitinase production was less. This probably is because M9C medium has a higher buffer capacity. Chitinase production is sensitive to the initial medium pH and to changes in pH during cell growth (data not shown; M. Young, Ph.D. thesis, University of California, Davis, 1981). The pH change during growth in M9C (0.3 pH units) was less than in Reese medium (1.0 pH unit).

Isolate IMR-R1 was chosen from the ultraviolet mutagenesis step, and it was mutagenized with ethyl methane sulfonate (0.3% survival). Isolate IMR-1E1 was obtained which produced a much larger zone of clearing than IMR-R1 and QMB1466 (Fig. 2). It also overproduced chitinase in M9C medium (Table 1). One round of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (25% survival) was done, but no isolates outproducing IMR-1E1 were obtained.

Mutation and selection of nonproducing mutants can result in production of higher-producing mutants than those produced by similar mutation and selection of the wild type (18). Eight chitinase nonproducing mutants were mutagenized with ultraviolet light, and a total of 4×10^9 cells were plated on M9C agar plates. Thirty-five revertants were obtained, but the maximum chitinase production achieved by any revertant was 80% of wild type (data not shown).

Maximum chitinase production. Strains QMB1466, IMR-R1, and IMR-1E1 were compared for maximum chitinase production. Figure 3 shows the chitinase activity of culture supernatants sampled over several days of growth in Reese medium without yeast extract. The max-

TABLE 1. *Mutagenesis and selection of strains of S. marcescens which overproduce chitinase*

Step	Mutagen[a]	Parental strain	No. of exp.	Survivors plated	Isolates screened on NAC plate	Isolates screened in shake flasks	Isolates	Chitinase production of isolate[b] (U/ml)
1	UV	QMB1466	8	14,763	615	39	IMR-R1	0.31
2	EMS	IMR-R1	3	4,148	300	3	IMR-1E1	0.44
3	NTG	IMR-1E1	1	1,029	100	2	IMR-NTG1	0.37

[a] UV, Ultraviolet; EMS, ethyl methane sulfonate; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.
[b] Assayed using the agar diffusion assay on supernatants from M9C (without yeast extract) shake flask cultures. Wild-type chitinase production was 0.21 U/ml.

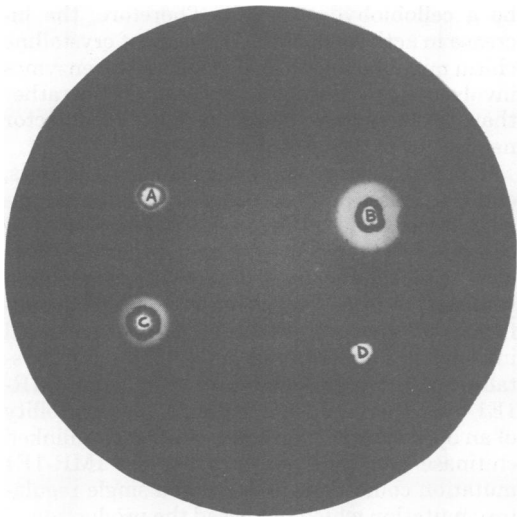

FIG. 2. *Zones of clearing on an NAC chitin plate produced by strains of S. marcescens. Strains were stabbed onto a 1% chitin NAC plate and incubated at 30°C for 36 h. The strains are as follows: A, Wild type, QMB1466; B, chitinase overproducer, IMR-1E1; C, chitinase overproducer, IMR-R1; D, chitinase nonproducer, IMR-NP1.*

imum chitinase activity of strain IMR-1E1 was 0.483 U/ml which was 2.7 times the wild-type level in this experiment. In Reese medium with yeast extract the maximum production of chitinase by IMR-1E1 was 0.592 U/ml (SD = 0.004, $n = 2$) which was about twice the normal wild-type activity. However, in M9C medium, mutant IMR-1E1 produced 3.3 times as much chitinase in 24 h as the wild type produced in 3 days (data not shown).

In Reese medium the wild type yields 2.0 (SD = 0.05, $n = 3$) times more cells. On a per cell basis, strain IMR-1E1 made four times more chitinase than the wild type in Reese medium with yeast extract.

Induction of chitinase enzymes. Endochitinase and chitobiase activities appear at similar times in the presence of chitin (Fig. 4). Neither enzyme was induced by glucose or mannose (data not shown). In late log phase or early stationary phase, chitobiase activity (NPGlu assay) was found both in the culture supernatant and on the surface of washed cells of both QMB1466 and IMR-1E1 (data not shown).

Crystalline chitin hydrolysis and chitobiase activity of IMR-1E1. Chitinase assays based on NAG production or reducing sugar production or on hydrolysis of chitin suspended in agar were used to demonstrate that IMR-1E1 overproduces chitinase. These assays primarily measure endochitinase activity since (i) the substrate is swollen chitin, crystalline chitin hydrolysis is not required for its degradation (11), and (ii) the chitobiase activity of the *S. marcescens* chitinolytic enzyme complex probably is in excess of the endochitinase activity as suggested by the fact that during chitin hydrolysis the primary hydrolysis product is NAG and little chitobiose is detected (S. Revah-Moiseev, M.S. thesis, University of California, Davis, 1978). Therefore, it was of interest to compare crystalline chitin hydrolysis and chitobiase production by IMR-1E1 with the wild type.

Figure 4 shows that IMR-1E1 produces more chitobiase than the wild type. However, NPGlu was used as a substrate for these chitobiase assays, and it was possible that NPGlu may be hydrolyzed by other enzymes including the endochitinase. Monreal and Reese (11) had demonstrated the endochitinase was not active against chitobiose. We found that the ratio of chitobiase activities determined with NPGlu and chitobiose as the substrates was constant (data not shown). This was true for wild type and IMR-1E1 supernatants collected at several times during growth on chitin. This suggests that NPGlu is being specifically hydrolyzed by the chitobiase, and that NPGlu is a useful substrate for measuring chitobiase activity.

Table 2 shows that IMR-1E1 produces increased levels of all activities of the chitinolytic enzyme system. Within any experiment the percentage increases in the three activities for IMR-1E1 were similar.

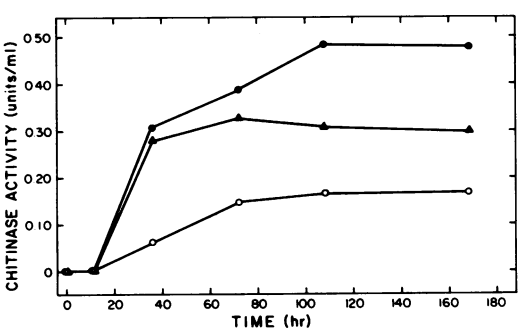

FIG. 3. *Time course of chitinase production. Cells were grown in Reese medium (pH 7.5) without yeast extract. The inocula were grown in LB medium and therefore were not induced for chitinase. The numbers of cells in the shake flasks after inoculation were: QMB1466 (wild type), 2.1 × 10^7 cells/ml; IMR-R1, 1.9 × 10^7 cells/ml; and IMR-1E1, 1.1 × 10^7 cells/ml. The numbers of cells during stationary phase were: QMB1466, 7.0 × 10^9 cells/ml; IMR-R1, 3.4 × 10^9 cells/ml; and IMR-1E1, 1.4 × 10^9 cells/ml. Chitinase activity of strains QMB1466 (○), IMR-R1 (▲), and IMR-1E1 (●) was determined by DNS assay.*

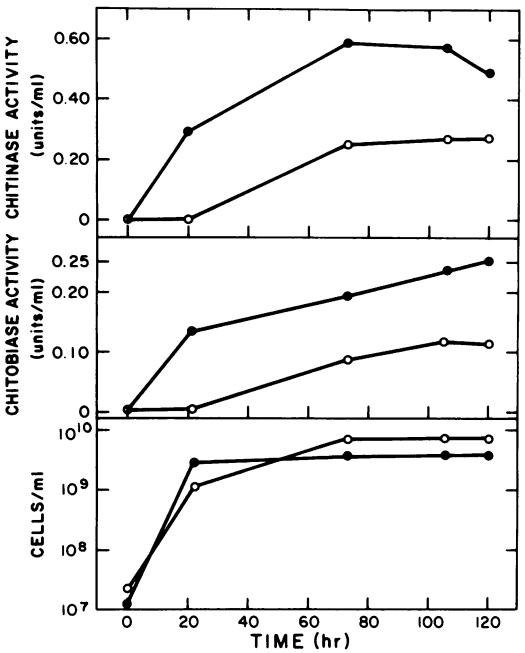

FIG. 4. *Simultaneous overproduction of chitinase and chitobiase enzymes by S. marcescens IMR-1E1. Replicate shake flasks containing 30 ml of Reese medium (pH 6.5) containing 0.5 g of yeast extract per liter were inoculated with cultures grown in rich LB medium (uninduced for chitinase). The number of cells per milliliter was counted with a microscope. Symbols: QMB1466* (○), *IMR-1E1* (●).

DISCUSSION

The mutant IMR-1E1 produces about two to three times as much chitinase activity as the wild-type *S. marcescens* QMB1466. The percentage increase varies with the medium composition, and the maximum value of chitinase production for IMR-1E1 (0.592 U/ml) could no doubt be further increased if fermentation conditions and medium composition were optimized.

The increase in activity against crystalline chitin for IMR-1E1 was proportional to the increase in activity against swollen chitin. Reese proposed a CH_1 factor required for activity against crystalline chitin because (i) the ratios of activity against crystalline and swollen chitin were different when different forms of chitin were used for chitinase induction and (ii) the ratio changed as the fermentation progressed (11). The CH_1 factor was analogous to the C_1 factor which had been believed to "activate" or deaggregate cellulose chains to initiate attack on crystalline cellulose (13). More recently a different model of cellulose degradation has been proposed in which the C_1 factor is suggested to be a cellobiohydrolase (5). Therefore, the increase in activity of IMR-1E1 against crystalline chitin might be due to an increase in the enzymes involved in hydrolysis of swollen chitin rather than to an increase in some additional factor needed for hydrolysis of crystalline chitin.

The fact that the crystalline chitin hydrolysis, endochitinase, and chitobiase activities all appear at the same time suggests the possibility of coordinate regulation of these activities. However, the time course of enzyme production would have to be followed more closely to confirm simultaneous appearance. The proportional increase in endochitinase, chitobiase, and crystalline chitin hydrolysis activities of strain IMR-1E1 over the wild type suggests the possibility of an operon or of coordinate control of unlinked chitinase genes. In either case, the IMR-1E1 mutation could be explained by a single regulatory mutation which increased the production of the enzymes equally. Alternatively, the IMR-1E1 mutation could be a tandem duplication of the chitinase genes. Tandem duplication has been shown to occur at reasonable frequencies in other enteric microorganisms (1, 2) and ethyl methane sulfonate can cause such duplications (7). If IMR-1E1 is a tandem duplication, then this would imply that the chitinase genes were linked on the chromosome, and the possibility of coordinate control would be neither supported or excluded. Preliminary results indicate that IMR-1E1 is quite unstable. Revertants producing near wild-type levels of chitinase often outnumbered IMR-1E1 cells in the final cell populations of IMR-1E1 cultures grown on Reese medium. Even in rich LB plus chitin medium, revertants are 10 to 20% of the final population. Such a high reversion rate is characteristic of tandem gene duplications (1).

Relatively little is known about the chitinolytic enzyme system of *S. marcescens*. The number and characteristics of the activities involved in chitin degradation, the chromosomal positions of the chitinase genes, whether or not the chitinase genes are coordinately controlled, and the identity of the inducer are some of the basic facts which are still unknown. Even the primary location of the chitobiase activity is unclear. Becker (Abstr. Annu. Meet. Am. Soc. of Microbiol. 1980, K133, p. 148) reported that chitobiase was not an exoenzyme, yet for the wild type and IMR-1E1 chitobiase activity was found on washed cells and in the supertant during cell growth, suggesting that at least some of the chitobiase is secreted. The reasons for these differences have not yet been resolved.

In conclusion, IMR-1E1 is a valuable strain for chitinase production. The nature of the IMR-1E1 mutation is not known, but its overproduc-

TABLE 2. *Production of enzymes of the S. marcescens chitinase system by strains QMB1466 (wild type) and IMR-1E1*

Enzyme	Substrate	Organism	Expt 1			Expt 2		
			Time[a] (h)	Enzyme activity (U/ml)	Ratio	Time[a] (h)	Enzyme activity (U/ml)	Ratio
Chitinase	Swollen chitin	IMR-1E1[b]	73	0.594	2.4	67	ND[c]	
		Wild type		0.247			0.206	
		IMR-1E1	106	0.572	2.2	93	0.589	3.1
		Wild type		0.264			0.191	
Chitobiase	NPGlu	IMR-1E1	73	0.187	2.3	67	ND	
		Wild type		0.081			0.087	
		IMR-1E1	106	0.238	2.0	93	0.248	3.6
		Wild type		0.120			0.073	
Chitobiase	Chitobiose	IMR-1E1				67	ND	
		Wild type					4.43	
		IMR-1E1				93	12.7	2.9
		Wild type					4.13	
Crystalline chitin hydrolysis activity	Milled chitin	IMR-1E1				67	ND	
		Wild type					0.066	
		IMR-1E1				93	0.361	3.5
		Wild type					0.103	

[a] Time of sampling after inoculation.

[b] Inocula for experiments 1 and 2 were grown overnight in LB medium and therefore were not induced for chitinase. In experiment 1 the average number of cells in replicate shake flasks after inoculation was: QMB1466, 2.2×10^7 cells/ml; IMR-1E1, 1.2×10^7 cells/ml. The number of cells at stationary phase was: QMB1466, 7.0×10^9 cells/ml; IMR-1E1, 3.4×10^9 cells/ml. In experiment 2 the average number of cells in replicate shake flasks after inoculation was: QMB1466, 2.2×10^7 cells/ml; IMR-1E1, 1.8×10^7 cells/ml. The number of cells at stationary phase was: QMB1466, 6.8×10^9 cells/ml; IMR-1E1, 3.5×10^9.

[c] ND, Not determined.

tion of several components of the chitinolytic enzyme system suggests several interesting possibilities.

ACKNOWLEDGMENTS

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