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Expression of multiple complex polysaccharide-degrading enzyme systems by marine bacterium strain 2-40

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Saprophytic marine bacterium strain 2-40 (2-40) can degrade numerous complex polysaccharides (CP) including agar, alginic acid, carrageenan, carboxymethylcellulose, chitin, β-glucan, laminarin, pectin, pullulan, starch, and xylan. The growth of 2-40 was assessed in minimal media containing one of 16 CP or simple carbohydrates, with the result that all supported growth. Each of the carbohydrase systems was elicited at highest levels by the homologous substrate. Each, excluding amylase, was repressed when 2-40 was cultured in glucose minimal synthetic media. Cyclic adenosine monophosphate alleviated the repression. Agarose as sole carbon source supported the synthesis of the most heterologous complex carbohydrase systems, although, generally, at a lower level of activity than the homologous CP.

Keywords: complex polysaccharide; glucose repression; hydrolytic enzymes; polysaccharidases

Introduction

Saprophytic marine bacterium strain 2-40 (2-40) was isolated from decaying salt marsh grass, *Spartina alterniflora*. It degrades numerous complex polysaccharides (CP) including agar, alginic acid, carrageenan, cellulose, chitin, glucan, laminarin, pectin, pullulan, starch, and xylan [1,29]. Thus, 2-40 can recycle a wide variety of plant, animal and microbial CP and is believed to play an important role in recycling carbon and nitrogen in the marine environment. Based upon phenotypic criteria, fatty acid analysis and 16S RNA sequence, 2-40 is proposed for placement in the recently named genus, *Microbulbifer* (submitted to IJSB), which includes degradative isolates such as shipworm symbionts [9].

Relatively little is known about the degradation of CP and regulation of CP-degrading systems by intact bacteria [21]. In most cellulolytic bacterial species, cellulase synthesis is repressed in the presence of readily metabolized carbon sources and induced by soluble, processed breakdown products of cellulose [4]. Similarly for agar, several agarase systems are induced by the homologous substrate and its breakdown products. Furthermore, some agarases have been shown to be repressed by glucose [9,20,23].

The molecular and genetic mechanisms of chitinase synthesis and the chitin degradative pathway have been well studied in *Vibrio furnissii* [3] as well as different species [8]. As is becoming apparent for CP degradation in general, this process in *V. furnissii* is very complex, involving at least three signal transduction systems and approximately 25 inducible proteins for substrate degradation [31] and each step is highly regulated. In general, however, the emerging paradigm for CP degradative enzyme systems is that they are induced by (modified) breakdown products of the substrate CP and repressed by soluble monomers such as glucose [8].

This study focuses on the expression of 10 CP-degrading systems of marine bacterium, strain 2-40 in minimal media containing a sole carbon source. Preliminary reports of this work have been presented at the 96th [28] and 97th [30] General Meetings of the American Society for Microbiology and a portion of this work was used in partial fulfillment of the PhD of LWE [29].

Materials and methods

Growth parameters

Bacterium 2-40 was cultured on Marine Agar 2216 (Difco, Detroit, MI, USA) or minimal media [MM; 2.3% (w/v) sea salts (Instant Ocean, Aquarium Systems, Mentor, OH, USA), 0.1% (w/v) yeast extract, 0.05% NH_4Cl (w/v) and 50 ml L^{-1} Tris-HCl, ph 7.6] containing a final concentration of 0.2% of one of the following carbon sources: agarose (low melting), d-galactose, alginic acid (low viscosity sodium salt), carrageenan (commercial; mixture of kappa and lambda), carboxymethylcellulose (sodium salt), colloidal chitin (crab shell; purified), glucosamine, N-acetyl-d-glucosamine, d-glucose, laminarin (from *Laminara digitata*), β-glucan, pectin (citrus fruit), pullulan (from *Aureobasidium pullulans*), starch (ACS Reagent), xylose, or xylan (from Beechwood). All chemicals were purchased from Sigma Chemical Co (St Louis, MO, USA), except agarose (FMC, Rockland, ME, USA). The complex carbohydrates were added to MM prior to autoclaving. The oligo- and mono-saccharides were prepared as 20% stocks in 20 mM Pipes buffer (pH 6.8), filter sterilized and added to cooled MM. The cultures were inoculated from starter cultures, grown overnight in the homologous MM, aerobically at 25°C. During cultivation, the OD_{600}, viable counts and direct counts were determined to assess the phase of growth. Unless otherwise noted, cultures were har-

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vested during the late logarithmic/early stationary phase growth transition.

Carbohydrase activity

For most experiments, whole cell and supernatant fractions were obtained by centrifugation at 11 000 × *g* for 15 min at 4°C. Culture fractions were partitioned into test aliquots and stored at –20°C until carbohydrase activity was assayed. Frequently, carbohydrase activity was determined using a modification [17] of the 3,5-dinitrosalicylic acid (DNSA) reducing sugar assay [24] Briefly, the enzyme preparations (0.3 ml) were incubated with 0.7 ml of CP substrates which were prepared as 0.5% stocks (except agarose; 0.2%) in either 0.025 M sodium citrate buffer (pH 5.0) or 0.01 M potassium phosphate buffer (pH 7.0). Carboxymethyl cellulose, chitin, laminarin and pectin were prepared in pH 5.0 buffer, agarose, alginic acid, carrageenan, pectin, pullulan, starch, and xylan, in pH 7.0 buffer. Agarose, alginic acid, chitin and carrageenan were boiled for 5 min to dissolve them prior to their addition to the reaction mixture. The reaction incubation times and temperatures were also substrate-dependent. Agarase, alginase, and xylanase activity reactions were incubated for 1 h at 25°C, while cellulase, chitinase, carrageenase, laminarinase, pectinase, pullulanase and amylase reactions were incubated for 2 days at 30°C. Color change in DNSA reagent was detected spectrophotometrically as it was reduced to 3-amino-5-nitrosalicylic acid by reducing sugars present in a reaction mixture.

For experiments investigating the alleviation of glucose repression, by cyclic adenosinemonophosphate, the enzymes were first concentrated by precipitation with 40% $(NH_4)_2SO_4$ (little activity was detected in the unprecipitated fraction) and an equally sensitive $K_3(Fe[CN]_6)$ reducing sugar assay was also used [23], with results comparable to the DNSA assay. This assay spectrophotometrically detects the color change in the $K_3(Fe[CN]_6)$ reagent (yellow) as it is reduced (colorless). Briefly, 100 μl of the enzyme preparation, 200 μl of Pipes buffer pH 6.8, and l00 μl of substrate (stock 0.8% prepared in Pipes buffer to give a final concentration of 0.2%) were vortexed and placed in a 45°C water bath for 1 h. Once removed, 20 μl of stop reagent (1% w/v $Na_2HPO_4 \cdot 7H_2O$, 1% w/v NaOH, pH 12.5) and 300 μl of color reagent (0.116% w/v $K_3(Fe[CN]_6)$, 0.22% w/v NaOH, pH 11.8) were added to the reaction mixtures, mixed, and placed in a boiling water bath for 3.5 min. The mixtures were then removed, cooled and the absorbance at 420 nm recorded.

For both reducing sugar assays and the protein assay, samples were measured in triplicate and the average taken. The average standard error (SE) was 0.284 with a maximum SE of 0.712. Negative controls were always included and contained the enzyme preparation, boiled for 5 min prior to addition to the reaction mixture. The values of these negative controls (which included reducing sugar in the media) were subtracted from the experimental values. The amount of reducing sugar was compared to glucose standard curves that were prepared freshly for each assay set. Enzymatic activities were reported as units of carbohydrase activity (μg reducing sugar generated ml^{-1} enzyme preparation h^{-1}) [2], or as specific activity (μg ml^{-1} units carbohydrase activity^{-1} μg ml^{-1} protein). Total protein concentrations in samples were measured using the Pierce BCA protein assay (Pierce Chemical Co, Rockford, IL, USA; BSA standard curve).

Effect of cyclic adenosine monophosphate on agarase repression

N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl cyclic AMP; db-cAMP) was used to facilitate entry of the cAMP into the cell. The db-cAMP stock was prepared just prior to use and was filter sterilized in 20 mM Pipes buffer, pH 6.8. A culture of 2-40 in MM + 0.2% d-glucose was harvested during early logarithmic phase and divided into four equal portions. One portion (control) was supplemented with 5.0 ml 20 mM Pipes buffer, pH 6.8; the second, with 100 μl db-cAMP + buffer; the third, with 100 μl db-cAMP + 0.2 % agarose; and the last with 0.2% agarose. Each of the four flasks was sampled at 20-min intervals for 120 min.

Results

To assess the expression of carbohydrase activity of marine bacterium strain 2-40, growing on a sole carbon source, it was cultivated in MM containing a final concentration of 0.2% of one of the following 16 substrates. Monosaccharides included: glucose, d-galactose, glucosamine, *N*-acetyl-d-glucosamine (NAG), and xylose. CP included: agarose, alginic acid, carrageenan, carboxymethylcellulose (CMC), chitin, glucan, laminarin, pectin, pullulan, starch, and xylan.

Individual degradative enzyme systems were best induced by the homologous substrate. In every case maximum activity was detected in the supernatant fraction of cultures in the logarithmic/stationary phase transition and these values are reported in Table 1. Some enzyme systems, eg agarase, amylase and laminarase, were active when 2-40 was cultivated in most of the sole carbon CP. Others, eg carrageenanase, chitinase and xylanase, were not active when 2-40 was grown in more than 50% of the tested sole carbon CP. In fact, the data suggest that only derivatives of chitin could induce the chitinase system.

To utilize supernatants for potential bioremediation of mixed CP and/or biofilms, it was necessary to identify a defined medium or substrate that would allow expression of the highest activities of the most enzyme systems. Of the 16 sole carbon sources tested in MM, agarose supported the most heterologous CP-degrading systems with the highest average activities (Table 1). Nine of the ten CP degradative enzyme systems were expressed when 2-40 was cultivated in MM + agarose, however generally at lower activity than in the homologous CP. Only the chitinase system remained inactive.

Amylases were the only enzymes that had significant activity when 2-40 was grown in MM + glucose (Table 1), a medium which supported the most rapid growth and the highest cell yield.

Since it appeared that the mechanism of the glucose effect could involve catabolite repression, it was postulated that cAMP would relieve that control. In fact, the only culture, growing in MM + glucose, which had any agarase activity was that to which both cAMP and 0.2% agarose

Table 1 Units and specific activities of carbohydrases in culture supernatants of 2–40 grown in complex and simple carbohydrate sole-carbon substrates[a]

Substrate	Units and specific activities									
	Agarase	Alginase	Amylase	CMCase[b]	Carrageenanase	Chitinase	Laminarinase	Pectinase	Pullulanase	Xylanase
Glucose	0[c]	0[c]	814 (6.2)[d]	0[c]	0[c]	16 (0.1)	24 (0.2)	0	3	8
Agarose	525 (7.2)	194 (2.7)	805 (9.0)	251 (3.4)	101 (1.4)	0	134 (1.5)	36 (0.4)	311 (10)	46 (0.5)
Alginic acid	230 (1.4)	633 (4.1)	419 (2.6)	20 (0.1)	97 (0.6)	0	109 (0.8)	0	59 (0.3)	50 (0.3)
CMC[b]	37 (0.3)	27 (0.2)	35 (0.3)	352 (2.6)	0	0	0	34 (0.3)	0	81 (0.6)
Carrageenan	47 (0.4)	5	309 (2.7)	0	209 (1.9)	0	31 (0.3)	128 (1.2)	4	59 (0.6)
Chitin	244 (2.2)	0	823 (5.4)	168 (1.1)	0	244 (3.6)	211 (1.4)	0	615 (4)	3
Galactose	46	0	15	0	0	31	63	0	27	0
Glucan	110 (1.3)	0	764 (10.2)	165 (2.2)	0	0	268 (3.1)	100 (1.3)	714 (9.5)	162 (0.3)
Glucosamine	142 (2.2)	56 (0.7)	0	0	0	0	54 (0.7)	0	0	0
Laminarin	61 (0.6)	0	589 (5.6)	27 (0.3)	0	0	1046 (9.9)	0	0	0
NAG[e]	144 (1.3)	81 (0.8)	744 (6.1)	0	0	0	16 (0.1)	0	0	0
Pectin	67 (0.4)	18 (0.1)	510 (3.0)	38 (0.2)	0	11	99 (0.6)	405 (2.4)	50 (0.1)	67 (0.4)
Pullulan	172 (2.7)	0	730 (9.8)	15 (0.2)	0	0	0	0	362 (4.9)	0
Starch	311 (2.0)	227 (1.3)	812 (4.0)	113 (0.7)	55 (0.3)	0	40 (0.2)	0	41 (3.2)	0
Xylan	273 (2.7)	72 (0.8)	97 (0.7)	97 (1.1)	0	0	115 (1.0)	9 (0.1)	0	358 (3.3)
Xylose	285 (1.6)	662 (3.7)	542 (2.1)	21	0	0	0	0	0	0

[a]Unless otherwise noted, supernatants were harvested from cultures during the logarithmic/stationary phase transition (as determined by optical density, direct cell counts and plate counts) of 2-40 (grown in minimal media containing 0.2% of the respective substrate). The fractions were assayed for the units of carbohydrase activity using the DNSA assay. Units of carbohydrase activity are reported as 1.0 μg of reducing sugar produced ml^{-1} under defined conditions (see Materials & Methods section).
[b]CMCase, carboxymethylcellulase; CMC, carboxymethylcellulose.
[c]Activity was measured from logarithmic phase cultures of 2-40 using the Ferric cyanide assay (see Materials & Methods section).
[d]Parenthetical values are the specific activities (units of carbohydrase activity μg^{-1} protein [determined using the BCA protein assay]). All values were rounded to the nearest 0.1 unit μg^{-1} protein (zero values not shown).
[e]NAG, N-acetyl-d-glucosamine.

were added (Table 2). Forty minutes after the addition of both cAMP and agarose, there were detectable levels of agarase on the cell surface (not shown), followed by activity in the medium 20 min later.

Discussion

This paper reports the degradation of 10 CP by marine bacterium, strain 2-40. Each enzyme system generally requires a number of enzymes with different specificities, functioning in concert to depolymerize the CP and yield monosaccharides [4,10,12,21]. For example, 2-40 synthesizes $\geq$ four agarose depolymerases in addition to $\geq$ one neoagarotetrosease and $\geq$ one neoagarobiosease [23,28; manuscript in preparation]. The enzymes are specialized to act upon different bonding arrangements and/or specific tertiary and quaternary structural domains (ie crystalline and amorphous regions [4]). While the complexities of stereochemical variance and bonding dictate that most enzymes be specific for a given oligosaccharide portion and/or domain, it is conceivable that some hydrolases may act on more than one CP. Nevertheless, the ability to synthesize more than 10 CP degradative systems represents a substantial genetic and physiological commitment on the part of 2-40.

Table 2 Effect of cAMP on agarase expression

MM + glucose[a] amendment	Agarase activity[b] Time post supplement (min)						
	0	20	40	60	80	100	120
Pipes buffer	–	–	–	–	–	–	–
100 μM cAMP	–	–	–	–	–	–	–
100 μM cAMP + 0.2% agarose	–	–	+	++	++	+++	+++
0.2% agarose	–	–	–	–	–	–	–

[a]2-40 grown in MM + 0.2% glucose.
[b]Units reducing sugar ml^{-1} supernatant were determined by the $K_3(Fe[CN]_6)$ assay: –, 0–20; +, 20–50; ++, 51–100; +++, >100.

As is the case for cellulases and other CP-degrading enzyme systems in other bacterial species [4], for 2-40, products of the homologous CP were inducers and soluble, metabolizable sugars were repressors. The repression of CP carbohydrase synthesis by monomeric, dimeric, or oligomeric substrates is common in bacteria [11], although in most cases the mechanisms remain unknown [5,15,22]. Repressive mechanism(s), at the transcriptional level, may resemble the well-characterized cAMP-mediated system of repression, or may control enzyme activity at the level of secretion [20].

Glucose (catabolite) repression inhibits full expression of microbial genes specifying proteins catabolizing less-readily usable substrates [13–16,18,32]. Except for amylase and possibly xylanase, glucose repressed all 2-40 carbohydrases tested, including agarase, alginase, CMCase, carrageenanase, chitinase, laminarinase, pectinase and pullulanase. Since the repression of agarase was relieved by cAMP, 2-40 CP-degradative enzyme systems are probably regulated by classical catabolite repression.

When CMC and laminarin were sole carbon sources, they repressed (or did not significantly induce) heterologous carbohydrase systems, with the exception of amylase production in laminarin. Nor did pullulan MM support

other heterologous carbohydrase systems, excluding amylase and agarase. Interestingly, glucose is the final hydrolysis product of CMC, laminarin, and pullulan.

The homologous CP induces because basal levels of enzyme (100–1000 times lower than induced levels) yield small quantities of oligosaccharides which serve as effectors or effector precursors [22]. The oligosaccharides are small enough to enter the cell, resulting in gratuitous enzyme production by 'inactivating' an operon repressor [7,11,16,18,25,32]. Often, a disaccharide is the actual molecule that is involved [12,19,26]. With the exception of amylase and pullulanase, results clearly show that all carbohydrases tested were best induced when 2-40 was cultured on the homologous CP sole carbon source substrate.

A relatively low level of carrageenanase activity is detected when 2-40 is grown in the homologous substrate. Carrageenan may be an auxiliary metabolite or carrageenanases may be invasions. For example, algal cell walls are comprised of carrageenan and agar. Low levels of carrageenanase activity may expose the agar to agarases.

Apparently, with at least ten regulated enzyme systems that degrade CP, a considerable amount of the 2-40 genetic machinery is devoted to substrate utilization, making the species a versatile saprophyte in nature, with the potential to recycle a variety of marine plant and animal polymers in dynamic ecosystems [6]. It may also be a potentially useful species for the bioremediation of CP [27].

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