Pullulanase Synthesis in *Klebsiella (Aerobacter) aerogenes* Strains Growing in Continuous Culture

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1. Pullulanase synthesis was studied in 16 classified (N.C.I.B.) strains and in an industrial strain (R) of *Klebsiella aerogenes* grown in chemostats containing maltose as inducer and sole carbon source. 2. Maximum synthesis was associated with carbon-limited growth at a low dilution rate (about 0.2 h^{-1}). The enzyme remained firmly cell-bound and seemed to be located on the cell surface. 3. Three strains had high activity (R, N.C.I.B. 5938, 8017), twelve were intermediate, and two (N.C.I.B. 8153, 9146) had negligible activity but were inducible with pullulan. 4. Pullulan similarly induced low, but adequate, activity in the other strains in conditions (nutrient limitation other than carbon-limitation) in which pullulanase was otherwise very seriously repressed. Nevertheless, in carbon limitation pullulan induced no more enzyme than did maltose, maltotriose or oligosaccharide mixtures, and 'hyperactivity' never developed on protracted culture. 5. Cyclic AMP relieved the transient repression produced by adding glucose to maltose-limited cultures and a further change to glucose-limited conditions led to constitutive pullulanase synthesis. 6. Amylomaltase and α -glucosidase activities were also examined but in less detail. 7. The presence of pullulanase in maltose-limited growth is discussed, but no clear function can be assigned to it at present. The molar growth yields for all the strains were very similar, and no correlation was found between the overgrowth of one strain by another and pullulanase activity. Further, any function as a general branching enzyme in polysaccharide synthesis seems unlikely.

Pullulanase hydrolyses pullulan, an α -glucan elaborated by *Pullularia pullulans* (Bender & Wallenfels, 1961; Catley, 1971), into maltotriose units, although traces of higher polysaccharides have also been detected (Drummond *et al.*, 1969). Because of its specificity for α -1,6-glycoside linkages, provided an α -1,4 linkage is immediately adjacent on each side (French & Abdullah, 1966), it has proved valuable in the analysis of starch-like polysaccharides (Manners, 1966; Mercier *et al.*, 1972) and as an industrial de-branching agent (Hathaway, 1970; Enevoldsen, 1971). Initially discovered (Bender & Wallenfels, 1961) in an unclassified *Klebsiella aerogenes* strain, pullulanase has also been reported in *K. aerogenes* A.T.C.C. 9621, N.C.I.B. 8021 (Brown *et al.*, 1965), *Streptococcus mitis* (Walker, 1968), *Streptomyces flavochromogenes* (Yagisawa *et al.*, 1972) and in strains of *Corynebacterium*, *Aeromonas*, *Flavobacterium*, *Vibrio*, *Actinoplanes* and *Streptosporangium* (Masuda & Sugimoto, 1971), and Ueda & Nanri (1967) isolated a pullulan-hydrolysing 'isoamylase' (discussed below) from *Escherichia intermedia*. Except with *S. mitis*, which synthesized pullulanase constitutively, maltose, maltotriose, pullulan, soluble starch and starch hydrolysates have been variously used as inducers in carbon-limited conditions. *K. aerogenes* pullulanase has been investigated and used much

more extensively than any other; nevertheless, its distribution in *K. aerogenes* strains in general, particularly in classified strains, has not been reported. For this reason we have determined the precise conditions necessary for optimum pullulanase induction in 16 N.C.I.B. strains of *K. aerogenes* growing in carbon-limited chemostat culture, in which, in contrast with batch culture, the enzyme remains firmly cell-bound. Its induction by pullulan, in conditions in which the enzyme in maltose- and maltotriose-limited organisms suffers very severe repression, is also reported, and an experimental procedure leading to constitutive pullulanase synthesis is described. The synthesis of other maltose-induced enzymes has also been studied, but in less detail. Part of this work has already appeared as a preliminary communication (Dean *et al.*, 1971).

Experimental

Organisms and culture

K. aerogenes N.C.I.B. 418 has been used in this laboratory for 30 years. The other N.C.I.B. strains (Table 1) were obtained from Torry Research Station, Aberdeen, U.K., and the Lord Rank Research Centre, High Wycombe, Bucks., U.K. kindly supplied strain R and the Bender & Wallenfels (1961) strain of *K.*

aerogenes. The strains were maintained by daily subculture in maltose-limiting medium at 30°C under aerobic conditions and, except where otherwise stated, were not introduced into chemostats until they had received at least 20 subcultures. Continuous culture was also carried out at 30°C, and air at 1 litre/min passed through the culture vessel (300ml working volume). This maintained the O₂ tension, measured with a New Brunswick O₂ probe, considerably in excess of the value [2kPa (15mmHg)] that Harrison & Pirt (1965) considered necessary for the balanced aerobic growth of *K. aerogenes*. The pH was maintained at 7.1±0.05 by a Pye autotitrator and at the low population density used (about 0.12mg dry wt. of organisms/ml) antifoaming agents were unnecessary. The chemostat, the general methods for its operation and the method for determining bacterial dry weight are described by Dean & Rogers (1967).

Solutions

Maltose-limited minimal medium contained, per litre: FeSO₄·7H₂O, 0.2mg; MgSO₄·7H₂O, 0.039g; (NH₄)₂SO₄, 0.96g; KH₂PO₄, 1.14g; Na₂HPO₄·12H₂O, 6.13g; maltose, 0.3g (chemostat culture) or 2.3g (batch culture); the pH was 7.1. AnalaR chemicals were used except for the maltose which was at least 99% pure. Maltotriose, pullulan and two oligosaccharide mixtures (partial acid hydrolysates of wheat starch), kindly supplied by the Lord Rank Research Centre, was used at the same concentration as maltose. The oligosaccharide preparations contained respectively: glucose, 1.5 and 2.1%; maltose, 2.5 and 2.9%; maltotriose, 3.0 and 3.5%; maltotetraose, 4.1 and 4.7%; maltopentaose, 4.2 and 4.7%; maltohexaose, 0.5 and 0.9%; the remainder was divided (approximately 1:2) between oligosaccharides of DP12 and DP40 (DP means degree of polymerization). Phosphate buffer (pH7.1) contained, per litre: KH₂PO₄, 2.96g; Na₂HPO₄·12H₂O, 16.0g; enriched when necessary with NaCl, 5.8g or MgSO₄·7H₂O, 0.063g and FeSO₄·7H₂O, 0.3mg. Citrate-phosphate buffer (pH5.0) contained, per litre: citric acid monohydrate, 10.2g, and Na₂HPO₄·12H₂O, 36.9g. Dinitrosalicylic acid reagent was prepared by mixing finely ground 3,5-dinitrosalicylic acid (1g) in rapid succession with water (10ml), 2M-NaOH (20ml) and water (25ml), cooling to room temperature (20°C), adding sodium potassium tartrate (30g) and diluting to 100ml with water. For amylose solution, amylopectin-free amylose (0.6g) suspended in ethanol (5ml) was added to 0.1M-NaOH (90ml) at 90°C and the residual amylose washed in with two further 1ml portions of ethanol.

Assays

Pullulanase. Bacteria, separated from the growth medium by centrifugation for 5–10min at 8000g,

were washed with citrate-phosphate buffer and resuspended in it at a biomass of 0.3–0.4mg dry wt./ml. Then 5ml of the suspension was incubated at 30°C in a thermostatically controlled water bath and 1.25ml of an aqueous solution of pullulan (50mg/ml) added with vigorous shaking. At intervals, 1ml samples were removed, added to 3ml of dinitrosalicylic acid reagent, heated in a boiling-water bath for 10min before final quenching and cooling by adding 10ml of water, and the extinction was read at 520nm in a Unicam SP.600 spectrophotometer against a blank withdrawn at zero time. Pullulanase specific activity was determined from the initial rate of the reaction and expressed as μmol of maltotriose liberated/min per mg dry wt. of organisms. When necessary, the organisms were disrupted by ultrasonic treatment for 5min at 0°C in an MSE-Mullard ultrasonic disintegrator. Agreement to within experimental error was obtained between the dinitrosalicylic acid assay and the neocuproin (Dygert *et al.*, 1965) method for determining reducing sugars. Both were superior to the Nelson (1944) method but the dinitrosalicylic acid assay was easier to use and was adopted. The medium in which the organisms had grown was similarly assayed after dialysis against distilled water for 2 days to remove interfering substances.

α-Glucosidase. Dean & Rodgers' (1969) technique, with *p*-nitrophenyl α-D-glucoside as substrate, was rigidly adhered to, and α-glucosidase specific activity expressed as nmol of *p*-nitrophenol liberated/min per mg dry wt. of organisms.

Amylomaltase. Bacteria, separated as described above, were washed twice with phosphate buffer (enriched with Fe²⁺ and Mg²⁺, pH7.1), resuspended in the same medium at a biomass of 0.8–0.9mg dry wt./ml, and 6ml of the suspension was ultrasonicated at 0°C for 6min to inactivate any α-glucosidase present. To a 5ml sample, maintained at 30°C, was added with vigorous shaking 1.25ml of an aqueous solution of maltose (18mg/ml), and 0.2ml samples were withdrawn at intervals; these were added to 1.8ml portions of water, heated for 2min at 100°C, then cooled in ice. Protein was precipitated with 1ml of NaOH (4.6mg/ml) and 1ml of ZnSO₄·7H₂O (20mg/ml) and the glucose in 2ml of filtrate determined by reaction with 8ml of Glucostat and Chromogen reagent (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). After 10min at room temperature, 2 drops of conc. HCl were added and the extinction was read at 400nm. During each assay an aqueous solution of glucose (2mg/ml) was treated in an identical manner and its extinction always agreed with a previously determined calibration curve. Amylomaltase specific activity, determined from initial rates, was expressed as nmol of glucose liberated/min per mg dry wt. of organisms.

α-Amylase. The bacteria were washed and resuspended in phosphate buffer (enriched with NaCl,

Table 1. *Pullulanase specific activities of K. aerogenes strains*

The organisms were grown at $D = 0.2\text{h}^{-1}$ in maltose-limited chemostats until steady-state conditions were achieved. Specific activity is expressed as μmol of maltotriose liberated/min per mg dry wt. of organisms.

Strain	Specific activity		Strain	Specific activity	
	Intact	Disrupted		Intact	Disrupted
N.C.I.B. 418*	0.24	0.21	N.C.I.B. 8793*	0.33	0.27
N.C.I.B. 5938	0.86	0.81	N.C.I.B. 8805*	0.31	0.25
N.C.I.B. 8017	0.83	0.80	N.C.I.B. 8806*	0.21	0.23
N.C.I.B. 8021*	0.37	0.34	N.C.I.B. 9024	0.24	0.24
N.C.I.B. 8152*	0.20	0.08	N.C.I.B. 9025	0.19	0.24
N.C.I.B. 8153	<0.01	0.02	N.C.I.B. 9146*	<0.01	<0.01
N.C.I.B. 8258	0.39	0.34	N.C.I.B. 9261	0.36	0.28
N.C.I.B. 8267*	0.30	0.27			
N.C.I.B. 8595*	0.17	0.17	R	0.72	0.69

* Type 1 *K. aerogenes*; the others were type 2.

pH 7.1) and 1.25 ml of a mixture of 1 ml of amylose solution and 1 ml of HCl (3.65 mg/ml) was added to 5 ml of bacterial suspension at 30°C. Reducing sugars in 1 ml samples, withdrawn at intervals, were determined as in the pullulanase assay, and α -amylase specific activity was expressed as μmol of reducing sugar liberated/min per mg dry wt. of organisms.

Macromolecular constituents. DNA, RNA, protein and polysaccharide were determined by methods based on the Dische, Bial, biuret and anthrone reactions respectively, as reported by Dean (1962, 1964).

Results and Discussion

Initial experiments with *K. aerogenes* N.C.I.B. 418

Maltose, maltotriose, pullulan and the oligosaccharide mixtures described above were equally effective inducers of pullulanase in carbon-limited conditions, and in the work to be reported maltose was the sole carbon source, except where stated otherwise. Optimum enzyme synthesis occurred at 30°C and the pullulanase specific activity of the organisms passed through a maximum value at a dilution rate (D) of 0.2h^{-1} , the activity at $D = 0.1, 0.3, 0.4, 0.5$ and 0.6h^{-1} being respectively 38, 79, 52, 27 and 10% of this value. The enzyme was tightly cell-bound; no activity was detected in the culture medium. Clarke *et al.* (1968) interpreted maxima in dilution rate-activity profiles in terms of a competition between increasing induction and increasing catabolite repression as D is increased but clearly a constant rate of induction and a minimum in repressor concentration would also suffice (see below). However, cyclic AMP (1 g/litre) did not prevent the decrease in pullulanase specific activity expected from a change in D from 0.2 to 0.5h^{-1} . The concentration of the nucleoside in a culture at $D = 0.2\text{h}^{-1}$ was gradually increased from 0 to 1 g/litre over 3.5 h and D then maintained at 0.4h^{-1} for 5 h followed by 3 h at 0.5h^{-1} .

Glucose-limited organisms, which exhibited no detectable activity, rapidly synthesized pullulanase in maltose-limited conditions. The steady-state specific activity was reached after 3–4 generations in chemostat culture after one subculture in batch culture, which represents a total of about 10 generations of growth in maltose-limited medium. This steady-state activity was constant from one chemostat experiment to the next, the value obtained at $D = 0.2\text{h}^{-1}$ lying in the range 0.20–0.27 unit (μmol of maltotriose liberated from pullulan/min per mg dry wt. of organisms).

Other *K. aerogenes* strains

Strain R and two N.C.I.B. strains (5938, 8017) had a high pullulanase specific activity, two had negligible activity (8153, 9146), and the remainder were intermediate, like N.C.I.B. 418 (Table 1). For comparison, the activity of the unclassified strain used in the original work on pullulanase (Bender & Wallenfels, 1961) was 0.57 unit in intact organisms and 0.53 unit in disrupted organisms, which is lower than that in our 'high-activity' strains. Maltose-limited *Escherichia coli* K12 contained no pullulanase. Nevertheless, irrespective of pullulanase activity, the conversion of maltose into biomass was very similar with all the *K. aerogenes* strains, the steady-state molar growth yields being close to the value of 144 mg dry wt./mol of maltose repeatedly obtained with *K. aerogenes* 418. Again no pullulanase was detected in the growth medium, and apart from strain 8152, where disruption led to a marked decrease, intact and disrupted organisms had very similar activities, suggesting the location of the enzyme on the cell surface. Further, adding formaldehyde, a strong inhibitor of permeases (Koch, 1963), during the assay decreased both activities in a parallel manner (Table 2), which contrasts with its rapid inhibition at 0.25 M concentration of β -galactosidase permease and its innocuousness towards β -galactoside hydrolase, as measured by the

Table 2. *Effect of adding formaldehyde during the pullulanase assay*

K. aerogenes N.C.I.B. 5938 was grown in maltose-limited medium at $D = 0.2\text{h}^{-1}$ until steady-state conditions prevailed. Enzyme activity is expressed as a percentage of the control value and the final formaldehyde concentration in the assay mixture is reported.

Formaldehyde (M)	Pullulanase specific activity (%)	
	Intact	Disrupted
0	100	100
0.03	67.3	52.5
0.06	39.0	45.5
0.09	29.7	26.8

activity in intact and disrupted organisms respectively (Smith & Dean, 1972).

The 'high-activity' strains (R, 5938, 8017), two intermediate strains (418, 8258) and the two strains with negligible activity (8153, 9146) were examined more extensively. Dilution rate-activity profiles showed that maximum pullulanase synthesis occurred at $D = 0.27, 0.25, 0.17$ and 0.20h^{-1} with strains R, 5938, 8017 and 8258 respectively and this, together with the maximum at $D = 0.2\text{h}^{-1}$ reported above for strain 418, determined the choice of $D = 0.20\text{h}^{-1}$ as a reasonable compromise for the experiments that follow. It was also established that, as at $D = 0.2\text{h}^{-1}$, pullulanase synthesis in strains 8153 and 9146 was still negligible at $D = 0.1, 0.3, 0.4$ and 0.6h^{-1} , the last value being close to the highest dilution rate obtainable at 30°C . Nevertheless, the enzyme was inducible by pullulan. After the changeover in the medium supplied to a chemostat culture of *K. aerogenes* 9146 (already in the steady state) from maltose-limited medium to pullulan-limited medium, the culture biomass remained constant, and in the new steady state the pullulanase specific activity was 0.36 unit in both intact and disrupted organisms. The slower response of strain 8153 is shown in Fig. 1. The biomass decreased for about 20h, then settled at about 75% of the maltose value, and the steady-state pullulanase activity was only 0.07 unit in intact organisms and 0.14 unit in disrupted organisms.

The α -glucosidase specific activities varied less from strain to strain than the pullulanase specific activities. There was no correlation between them (Table 3) nor between pullulanase activity and total polysaccharide content, although three strains had a higher content (up to 10% of the dry wt.) than the rest. Strain 418 had a lower DNA content and strain 8017 a higher RNA content than the other strains, but the protein contents were not very different (Table 4). No α -amylase activity was detected in tests on maltose-limited *K. aerogenes* 418 and 5938 nor in the Bender

Fig. 1. *Utilization of pullulan as sole carbon source by K. aerogenes N.C.I.B. 8153*

At zero time the medium feed to a chemostat culture at $D = 0.2\text{h}^{-1}$ was changed from maltose-limiting to pullulan-limiting medium. O, Actual biomass; ----, calculated biomass, assuming pullulan was not utilized. The calculation was based on the relation: $C_t = C(1 - e^{-Dt})$, where C_t and C are, respectively, the concentrations of pullulan in the culture vessel and in the medium reservoir; D is the dilution rate and t the time after the changeover in the medium supply.

Table 3. *Steady-state α -glucosidase specific activities of K. aerogenes strains*

Intact organisms, taken from maltose-limited chemostats at $D = 0.2\text{h}^{-1}$, were assayed. Specific activity is expressed as nmol of *p*-nitrophenol liberated/min per mg dry wt. of organisms.

Strain	Specific activity
N.C.I.B. 418	33
N.C.I.B. 5938	40
N.C.I.B. 8017	43
N.C.I.B. 8153	45
N.C.I.B. 8258	50
N.C.I.B. 9146	46
R	30

& Wallenfels (1961) strain. Possibly the very low activities, which are disadvantageous in carbohydrate structural analysis by pullulanase (Mercier *et al.*, 1972), are not detected in our assay.

Induction in 'repressed' conditions

Changing the carbon source from maltose to pullulan also induced pullulanase in the conditions (i.e.

Table 4. *Macromolecular composition of K. aerogenes strains*

Conditions of growth were as in Table 1. The values are expressed relative to those for *K. aerogenes* N.C.I.B. 418, which contained 0.09, 0.14, 0.70 and 0.031 mg/mg dry wt. of DNA, RNA, protein and total polysaccharide respectively.

Strain	DNA	RNA	Protein	Polysaccharide
N.C.I.B. 418	1.0	1.0	1.0	1.0
N.C.I.B. 5938	1.4	0.9	1.0	1.0
N.C.I.B. 8017	1.3	1.4	1.1	2.8
N.C.I.B. 8153	1.2	0.8	1.1	3.2
N.C.I.B. 8258	1.5	0.8	1.1	3.3
N.C.I.B. 9146	1.5	0.9	1.0	1.1
R	1.6	0.9	1.2	0.8

nutrient limitation other than carbon limitation) in which the enzyme in pullulanase-positive strains was severely repressed. For example, the steady-state pullulanase specific activity of *K. aerogenes* 5938 was zero when grown in NH_4^+ -limited medium containing a 100% excess of maltose, compared with 0.033 unit in the corresponding pullulan medium. The latter is only about 3% of the activity in maltose-limited organisms. The synthesis of 'excess' of polysaccharide, which is associated with NH_4^+ -limited growth (Tempest & Dicks, 1967), was also much decreased, presumably owing to the slow degradation of pullulan resulting from the low activity. Nevertheless, some accumulation did occur, since the organisms contained 0.06 mg of polysaccharide/mg dry wt. compared with 0.03 mg/mg in maltose-limited organisms and 0.29 mg/mg in NH_4^+ -limited maltose organisms. As in Fig. 1 the biomass again decreased initially, but only for 12 h before increasing again and settling at 70% of the maltose value. Essentially similar results were obtained with *K. aerogenes* 418, and further experiments revealed that although a 100% excess of maltose prevented pullulanase synthesis in strains 418 and 5938, a 200% and a 400% excess were respectively necessary for the complete suppression of α -glucosidase synthesis.

The mechanism underlying this induction of pullulanase by pullulan but not by maltose is not clear. The less than exponential decline in biomass after the changeover in the medium feed shows that pullulan was used from the start, as might occur if mutants immune to catabolite repression gradually replaced the original population. However, as the very low steady-state activity shows, the relief from repression was slight. The development of activity in the pullulanase-negative strains described in the preceding section could be similarly attributed to an overgrowth by (pullulanase-positive) mutants, but the absence of any change in biomass when *K. aerogenes* 9146 was used implies their presence initially in

appreciable proportions. A calculation shows that a specific activity of 0.016 unit would provide sufficient maltotriose to maintain the entire culture at $D = 0.2 \text{ h}^{-1}$, but since pullulanase is assayed at its optimum pH of 5.0 and the organisms were cultured at pH 7.1, the necessary activity is probably higher. Between 5 and 10% of mutants of specific activity 0.36 unit (the observed steady-state activity) must therefore have been present in the maltose culture, and their action on pullulanase should have been detected in the enzyme assay. The alternative, a response by most of the organisms, raises the question of the identity of the inducer of pullulanase, since the penetration of pullulan (mol.wt. 1.4×10^5 – 1.5×10^5) to the inducer sites in the DNA is unlikely. The location of the enzyme on the cell surface implies that, when pullulanase-producing organisms act on pullulan, degradation products (maltotriose and by further hydrolysis maltose) enter the cell, and the situation at the inducer sites would be the same as when maltose and maltotriose were the carbon sources. This explains why maltose and maltotriose are inducers, but does not explain why induction by pullulan, but not by maltose, can occur.

Amylomaltase

The complete repression of α -glucosidase in the NH_4^+ -limited conditions described above suggested the operation of another enzyme in the first step in maltose metabolism. In *E. coli*, amylomaltase liberates glucose from maltose by a transferase reaction in which maltodextrins are also produced (Schwarz, 1967) and on this basis a specific activity of 46 units would provide sufficient glucose to maintain our strains at $D = 0.2 \text{ h}^{-1}$. Steady-state 'amylomaltase' specific activities of 96 and 113 units were observed in NH_4^+ -limited and in maltose-limited *K. aerogenes* 5938 respectively, even though α -glucosidase synthesis was derepressed in the latter conditions (Table 3), but no attempt was made to confirm the production of maltodextrins. Glucose was not produced in the assay conditions in the absence of maltose, nor was maltose hydrolysed in the absence of the cell preparation. The use of disrupted organisms was obligatory. No activity was detected in intact organisms; presumably the liberated glucose was transformed into substances not detected in the Glucostat assay. Further, the rapid inactivation of α -glucosidase by ultrasonication compared with the stability of 'amylomaltase' made a distinction between these enzymes possible in maltose-limited organisms. The other strains were not examined, apart from maltose-limited organisms of strain 418, which had an 'amylomaltase' specific activity of 68 units.

Reference has already been made to the elaboration of large amounts of 'excess' of polysaccharide by NH_4^+ -limited organisms and some 20% more was

Scheme 1. Possible inter-relationship between amylomaltase and α -glucosidase in glycogen synthesis

The scheme is oversimplified since many chain-lengthening and branching steps take place before the glycogen molecule is synthesized.

produced from maltose than from glucose. Although some was undoubtedly slime polysaccharide, much is considered to be glycogen (Tempest & Dicks, 1967). Scheme 1 shows a possible inter-relationship between the maltose-degrading enzymes and glycogen biosynthesis, which might function exclusively or additionally to the mechanism operating when carbon sources other than maltose are used. We postulate that besides hydrolysing maltose, α -glucosidase also degrades the dextrins produced by amylomaltase action. When it is repressed, these dextrins act as 'primers' for glycogen synthesis, D-glucosyl residues being transferred to them by the action of glycogen synthetase as found by Greenberg & Preiss (1964) in their (unclassified) strain of *K. aerogenes*. The variety of substances which were active in tests on a purified glycogen synthetase preparation from *K. aerogenes* (A.T.C.C. 12658, N.C.I.B. 8806) makes this primer function, in principle, possible (Gahan & Conrad, 1968), but we ascribe an action to α -glucosidase (but not pullulanase) similar to that of maltodextrin phosphorylase in *E. coli* K12 (Schwarz & Hofnung, 1967). Clearly further work with cell-free preparations obtained by a gentler method of cell rupture is necessary.

Mixed carbon sources; 'constitutive' pullulanase

The medium supplied to a carbon-limited culture in the steady state of growth at $D = 0.2\text{h}^{-1}$ was changed from maltose-limiting medium to medium containing maltose and another carbon source,

carbon limitation still prevailing. With glucose as the second carbon source pullulanase synthesis was transiently repressed in *K. aerogenes* 5938 (Fig. 2). This effect has been confirmed several times, and although the repression was sometimes greater (Fig. 3a) it was never less than in Fig. 2. Moreover, as in the similar transient repression of β -galactosidase synthesis in (unclassified) *K. aerogenes* (de Crombrughe *et al.*, 1969), it was eliminated by cyclic AMP (Fig. 3b). It is difficult to be certain whether pullulanase synthesis was actually stimulated, since the scatter in the experimental points in Fig. 3(b) is unusually large, but it is clear that the ribose in the high concentration (1 g/litre) of cyclic AMP added was not utilized as a carbon source (Fig. 3c). However, the constancy of the culture biomass in Figs. 2 and 3(c) shows that both maltose and glucose were metabolized. Maltose plus succinate (3:1, w/w) and maltose plus sucrose (3:1, w/w) mixtures were similarly utilized, but with the former a transient repression was followed by a permanent repression to 80% of the initial activity. In contrast, on growth in maltose and sucrose medium, the steady-state pullulanase specific activity increased by 14% without any transient repression.

Pullulanase synthesis continued in the absence of an inducer when the organisms in the steady state in maltose plus glucose medium (Fig. 2) were subjected to a further change to glucose-limited conditions (Fig. 4). Severely prolonged repression occurred initially, but a steady-state activity, about 70% of the fully induced value, was achieved 200h after the changeover. *K. aerogenes* 418 responded in a similar

Fig. 2. Transient repression by glucose of pullulanase synthesis in *K. aerogenes* N.C.I.B. 5938

At zero time the medium feed to a chemostat culture at $D = 0.2\text{h}^{-1}$ was changed from maltose-limited medium (0.3 g of maltose/litre) to maltose plus glucose medium (0.15 g of each carbon source/litre). $\circ$, Pullulanase specific activity; $\bullet$, biomass.

Fig. 3. Elimination by cyclic AMP of glucose repression of pullulanase synthesis in *K. aerogenes* N.C.I.B. 5938

Conditions were as in Fig. 2, but the changeover in the medium supply was made at the time indicated by the arrow ($\downarrow$). In (b) cyclic AMP (2 g/litre) was added to the medium reservoir at zero time so that the concentration in the culture vessel would be 1 g/litre at $\downarrow$. Thereafter the cyclic AMP was maintained at 1 g/litre. (a) $\circ$, Control (no cyclic AMP); (b) $\bullet$, cyclic AMP present; (c) biomass.

manner, but the final activity was only 50% of the maltose-limited value. However, this constitutive pullulanase synthesis was as dependent on dilution

Fig. 4. Constitutive pullulanase synthesis in *K. aerogenes* N.C.I.B. 5938

At zero time the medium supply to a chemostat culture at $D = 0.2\text{h}^{-1}$ was changed from maltose plus glucose medium (carbon-limiting, 0.15 g of each carbon source/litre) to glucose-limiting medium (0.3 g of glucose/litre).

Table 5. Dilution rate-dependence of constitutive pullulanase synthesis

The results are expressed as a percentage of the maximum pullulanase specific activity obtained at $D = 0.2\text{h}^{-1}$.

D (h^{-1})	Pullulanase activity	
	<i>K. aerogenes</i> N.C.I.B. 418	<i>K. aerogenes</i> N.C.I.B. 5938
0.1	<6	64
0.2	100	100
0.4	53	91
0.6	<6	<6

rate as that induced in organisms grown only in maltose-limited medium, maximum activity again occurring at $D = 0.2\text{h}^{-1}$ (Table 5). This is understandable if the concentration of a repressor passes through a minimum value. von Meyenburg (1969) observed a minimum and then a maximum value in baker's yeast, and the catabolite repression of constitutive enzyme synthesis is well established (Brown & Monod, 1961; McFall & Mandelstam, 1963).

Culture stability

Unlike α - and β -galactosidases (Smith & Dean, 1972), pullulanase 'hyperactivity' did not develop during protracted chemostat culture. The pullulanase specific activities of *K. aerogenes* 418 and 5938,

obtained at 10 and at 127 generations in maltose-limited medium, were not significantly different, and were not increased by culture in the presence of the other inducers. In contrast, and although without effect on strain 418, very prolonged batch culture of strain 5938 in maltose-limited medium lowered the pullulanase specific activity subsequently obtained in chemostat culture. In three replicate experiments the activity had decreased to about 50% of the optimum value shown in Table 1 when 350–450 serial subcultures had taken place, but no reduction was evident at 300 subcultures. Activity was similarly developed in the pullulanase-negative strains. For example, the pullulanase specific activity of *K. aerogenes* 9146 was negligible in tests after 200 subcultures, but after 500 it had risen to 0.49 unit. Little is known about the underlying mechanisms, but it is feasible that the activities might converge towards a common value, possibly the stable activity of strain 418. Mixed-population studies, carried out before those changes had occurred, showed that when mixed in equal proportions strain 5938 outgrew strain 418 within 25–30 generations of maltose-limited chemostat culture at $D = 0.2\text{h}^{-1}$, but strain 9146 similarly outgrew strain 5938. The developing activity in strain 9146 cannot therefore be ascribed to cross-contamination by the other strains, though the possibility that the fall in activity in the strain 5938 cultures was due to the presence of strain 9146 is not eliminated. Rigorous preventive measures were taken, however, and the finding that the decline set in in replicate experiments after at least 2100 generations of growth (one subculture is about seven to eight generations), but never earlier, makes it unlikely.

Concluding remarks

Ryman & Whelan (1971) classified the enzymes that directly debranch glycogen and amylopectin, according to their ability or inability to hydrolyse pullulan, as pullulanases (*K. aerogenes* pullulanase, plant R-enzyme) or isoamylases (*Pseudomonas* isoamylase, baker's yeast isoamylase), the individual enzymes being further differentiated by the extent to which glycogen, amylopectin and their limit dextrins were attacked (Yokobayashi *et al.*, 1970; Gunja-Smith *et al.*, 1970; Evans & Manners, 1971; Lee & Whelan, 1971). On this basis the *E. intermedia* enzyme (see the introduction) is a pullulanase. Nevertheless, like *Pseudomonas* isoamylase, it completely hydrolysed starch and glycogen, which crystalline *K. aerogenes* pullulanase (Wallenfels & Rached, 1966) only partly debranched. Further, Ueda & Nanri (1967) found that pullulan induced much less enzyme activity than maltose or dextrin, which contrasts with our finding of equal effectiveness and with the marked superiority of starch hydrolysates in inducing pullulanase in *K. aerogenes* (unclassified) claimed by

C.P.C. International Inc. (1971). Although understandable when oligosaccharides containing appropriate glycosidic linkages are the carbon sources, the precise function of pullulanase in maltose-utilizing organisms is still not resolved. As established at present (Abdullah & French, 1970; Lee & Whelan, 1971), its synthetic capabilities appear to be limited to polymerizing maltose to maltosylmaltooses, maltotriose to maltotriosylmaltotrioses, and joining maltose to amylose and Schardinger α -dextrin by α -1,6 linkages. In non-repressing conditions, organisms practically devoid of pullulanase metabolized maltose at least as efficiently in our chemostats as those showing high activity, and its severe repression in the growth conditions in which the synthesis of 'excess' of polysaccharide was greatest argues against any general function as a branching enzyme in the process. Nevertheless, in pullulanase-producing organisms, its production in the appropriate conditions appears to be a stable property, particularly of *K. aerogenes* N.C.I.B. 418.

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