Physiological and biochemical changes accompanying the loss of mucoidy by *Pseudomonas aeruginosa*

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***Pseudomonas aeruginosa* M60, a mucoid strain, was grown in continuous culture (D 0.05 h⁻¹) under ammonia limitation with glucose as the carbon source. Steady-state alginate production occurred for only 1–2 d under these conditions [q_{alginate} 0.097 g alginate h⁻¹ (g dry wt cells)⁻¹], after which time the percentage of mucoid cells and the alginate concentration in the culture decreased in parallel and approached zero after approximately 10 d. These changes were accompanied by similar decreases in the activities of the alginate biosynthetic enzymes (represented by phosphomannomutase and GDP-mannose dehydrogenase) and by a large increase in the activity of the first enzyme of the 'external' non-phosphorylative pathway of glucose metabolism, glucose dehydrogenase. In contrast, the activities of other enzymes associated with this pathway (gluconate dehydrogenase, 2-ketogluconate kinase plus 2-ketogluconate-6-phosphate reductase) or with the 'internal' phosphorylative pathway of glucose metabolism (glucokinase and glucose-6-phosphate dehydrogenase) remained essentially unchanged. The loss of mucoidy and alginate production was accompanied by the appearance of low concentrations of intracellular polyhydroxyalkanoate (PHA) and of extracellular gluconate and 2-ketogluconate (partly at the expense of alginate production and partly as a result of increased glucose consumption). It is suggested that ammonia-limited, glucose-excess cultures of *P. aeruginosa* growing at low dilution rate are unable fully to regulate the rate at which glucose and/or its 'external' pathway metabolites are taken up by the cell, and therefore form copious amounts of alginate in order both to overcome the potentially deleterious osmotic effects of accumulating surplus intracellular metabolites and to consume the surplus ATP generated by the further oxidation of these metabolites. The loss of mucoidy invokes the use of an alternative, but analogous, strategy via which non-mucoid cells produce an osmotically inactive intracellular product (PHA) plus increased amounts of the extracellular metabolites gluconate and 2-ketogluconate via the low-energy-yielding and, under these conditions, largely dead-end 'external' metabolic pathway.**

Keywords: *Pseudomonas aeruginosa*, alginate production, glucose metabolism during loss of mucoidy, production of 2-ketogluconate and polyhydroxyalkanoate (PHA)

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen which commonly infects the lungs of cystic fibrosis patients. The organism typically becomes mucoid following infection

and secretes copious amounts of alginate, a viscous exopolysaccharide which is an important virulence factor and a major contributor to the mortality of these patients (May *et al.*, 1991). The ability to synthesize alginate enhances the colonial and survival properties of the organism by increasing its ability to form a biofilm and thus to protect itself from attack by antibiotics, an advantage that readily leads to the selection of mutants

Abbreviations: GMD, GDP-mannose dehydrogenase; PHA, polyhydroxyalkanoate; PMM, phosphomannomutase; PQQ, pyrroloquinoline quinone.

with the capacity to overproduce alginate (Deretic *et al.*, 1994).

Bacterial alginates are linear heteropolysaccharides composed of D-mannuronic acid linked to its C-5 epimer L-guluronic acid via (1-4)- β -glycosidic linkages; the mannuronic acid residues may be O-acetylated at the C-2 or C-3 positions (May *et al.*, 1991). Most of the genes encoding the enzymes that catalyse the synthesis of alginate from fructose 6-phosphate have been identified and characterized, viz. *algD* encoding GDP-mannose dehydrogenase (GMD), *algA* encoding the bifunctional enzyme phosphomannose isomerase/GDP-mannose pyrophosphorylase and *algC* encoding phosphomannomutase (PMM), together with *algE*, *algF* and *algG* encoding a membrane protein, an acetylase and an epimerase, respectively (Chitis & Ohman, 1993; Deretic *et al.*, 1994). Recent evidence suggests that GMD is a major kinetic-control point in alginate biosynthesis (Tatnell *et al.*, 1994).

Most of the genes encoding these biosynthetic enzymes, with the exception of *algC*, are organized into a gene cluster which shows evidence of operonic structure (Chitis & Ohman, 1993; Deretic *et al.*, 1994). The expression of *algD*, the first gene of the cluster, and also of *algC* is enhanced by a wide range of environmental signals similar to those encountered in the lungs of cystic fibrosis patients (e.g. high osmolarity, dehydration or nutrient limitation) as well as by various genetic changes (Deretic *et al.*, 1991, 1994; Sokol *et al.*, 1994). Transition to the mucoid state also occurs, albeit to a limited extent, even during growth under energy-limited conditions which, since alginate synthesis is highly energy-dependent, implies not only that alginate production is of major importance to the survival of the cell but also that carbon must be channelled into the central energy-conserving pathways of the cell as efficiently as possible (see Schlichtman *et al.*, 1994).

Alginate is synthesized at particularly high rates [0.27–0.44 g alginate h⁻¹ (g dry wt cells)⁻¹] during the growth of mucoid strains of *P. aeruginosa* under ammonia-limited conditions (Mian *et al.*, 1978) where it clearly imposes a major carbon and energy burden on the cell. Not surprisingly, therefore, most mucoid strains are relatively unstable outside the selective environment of the cystic lung, and revert relatively rapidly to the non-mucoid state.

Glucose metabolism has been extensively investigated in non-mucoid strains of *P. aeruginosa* and shown to occur by different routes depending on the nature of the growth environment. During growth in continuous culture under glucose-limited conditions the glucose is initially metabolized to 6-phosphogluconate via an 'internal' phosphorylative pathway catalysed by a glucose uptake system, glucokinase and glucose-6-phosphate dehydrogenase, whereas during growth under glucose-excess conditions it is metabolized via an 'external' non-phosphorylative pathway catalysed by a pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase and a flavin-dependent gluconate dehydrogenase (both located in the periplasm), followed by gluconate and 2-ketogluconate uptake systems, gluconate kinase, 2-ketogluconate kinase and 2-

keto-6-phosphogluconate reductase (Midgley & Dawes, 1973; Roberts *et al.*, 1973; Whiting *et al.*, 1976a, b). In contrast, little work has been carried out on glucose metabolism by mucoid strains of this organism.

This paper describes the nature of glucose metabolism in a mucoid strain of *P. aeruginosa* growing in ammonia-limited continuous culture with glucose as the carbon source, together with the physiological and biochemical changes that occur during the transition of this strain to a non-mucoid phenotype.

METHODS

Bacterial strains. *P. aeruginosa* strain M48, a mucoid clinical isolate from a cystic fibrosis patient, was obtained from Dr P. Gale, Public Health Laboratory, Leicester Royal Infirmary, UK. A slightly more stable mucoid form of M48 was isolated after repeated batch growth in a glycerol-mineral salts medium (see below) and designated M60. Both strains were stored in 20% (w/v) glycerol at -70 °C. Prior to use, each strain was streaked on to Luria broth agar supplemented with 60 mM glycerol and a single mucoid colony was picked off and used in the subsequent experiment.

Growth of *P. aeruginosa*. Batch culture was carried out at 30 °C (pH 7.0) in 500 ml baffled flasks containing 150 ml mineral salts medium C (Mian *et al.*, 1978), modified to contain higher concentrations of FeSO₄·6H₂O (10 mg l⁻¹) and (NH₄)₂SO₄ (3.0 g l⁻¹) with glycerol (4 g l⁻¹) as the carbon substrate. Continuous culture (*D* 0.05 h⁻¹) was carried out in a 2 l laboratory fermenter (LH Engineering 500 series, 1.8 l working volume) at 30 °C containing medium C modified to contain (NH₄)₂SO₄ (0.5 g l⁻¹) as the growth-limiting nutrient and glucose (10 g l⁻¹) as the carbon source. Iron was supplied separately as FeSO₄·6H₂O (0.21 g l⁻¹ stock solution acidified with 2 ml H₂SO₄) to maintain an input concentration of 10 mg l⁻¹. The pH of the medium was maintained at 7.00 ± 0.05 by the separate addition of 2 M KOH. Antifoam RD emulsion (Dow Corning) was diluted fivefold and added as required.

Preparation of washed cells, broken cells, high-speed membrane fraction and high-speed supernatant fraction. Cultures were diluted 20-fold with medium C lacking carbon and nitrogen, and the cells were separated from the alginate-containing culture supernatant by centrifugation at 16 000 *g* for 15 min. The pelleted cells were washed twice in medium C and finally resuspended to a concentration of 5 mg dry wt cells ml⁻¹ in 60 mM glycylglycine buffer (pH 7.1) (Ng & Dawes, 1973) prior to sonication on ice for 3 × 30 s at an amplitude of 12 μ . The broken cells were centrifuged at 8000 *g* for 10 min to remove cell debris, and 3 ml of the resultant cell-free extract was then centrifuged at 100 000 *g* for 2 h to produce a high-speed membrane fraction (HSMF) which was subsequently resuspended in 1 ml 60 mM glycylglycine buffer (pH 7.1) and used to assay glucose dehydrogenase and gluconate dehydrogenase, and a high-speed supernatant fraction (HSSF) which was used to assay the other enzymes of glucose metabolism. Alternatively, washed cells were resuspended to a concentration of 5 mg dry wt cells ml⁻¹ in 10 mM MOPS buffer (pH 7.0) supplemented with 2 mM DTT (Sá-Correia *et al.*, 1987), then sonicated and centrifuged as described above to produce a HSSF which was used to assay two of the alginate biosynthetic enzymes, GMD and PMM.

Enzyme assays. Glucose dehydrogenase and gluconate dehydrogenase activities were assayed in the HSMF using the

methods of Matsushita & Ameyama (1982) and Matsushita *et al.* (1982), respectively; in some experiments the HSMF was incubated with 12 μ M PQQ prior to assay of glucose dehydrogenase activity as described by van Schie *et al.* (1985). Glucokinase, glucose-6-phosphate dehydrogenase, and the Entner–Doudoroff enzymes 2-keto-3-deoxy-6-phosphogluconate aldolase and 6-phosphogluconate dehydratase were assayed as described by Cornish *et al.* (1988). Gluconate kinase, and 2-ketogluconate kinase plus 2-ketogluconate-6-phosphate reductase, were assayed as described by Whiting *et al.* (1976b). PMM and GMD were assayed by the method of Sá-Correia *et al.* (1987) except that 50 mM Tris/HCl buffer (pH 7.5) was replaced by 50 mM glycylglycine buffer (pH 8.5).

Mucoidy. The percentage of mucoid cells in the total cell population was measured by serially diluting the culture in sterile mineral salts medium lacking carbon and nitrogen, then spreading samples onto Luria broth agar plates supplemented with glycerol (6 g l⁻¹). Mucoid and non-mucoid colonies were easily distinguishable after incubation for 48 h at 30 °C.

Cell density. Cell density was determined spectrophotometrically for diluted culture and gravimetrically for washed cell suspensions as described previously (Williams *et al.*, 1994).

Measurement of substrate utilization and alginate production by washed cells. Washed cells resuspended in glycylglycine buffer (60 mM, pH 7.1) at a density of 1 mg dry wt cells ml⁻¹ were incubated at 30 °C in a stirred vessel with a final volume of 6 ml. Glucose, gluconate or 2-ketogluconate (all 40 mM) were added after temperature equilibration, then samples (1.5 ml) were withdrawn at appropriate time points and centrifuged rapidly at 11600 *g* for 10 min at 4 °C to remove cells. The resultant supernatant was assayed for glucose, gluconate, 2-ketogluconate and alginate as described below.

Electron microscopy. Sedimented cells washed in 50 mM potassium phosphate buffer (pH 6.8) were fixed in buffered 2.5% (w/v) glutaraldehyde, then washed in 50 mM potassium phosphate buffer (pH 6.8) and post-fixed in 1% (w/v) osmium tetroxide in 50 mM potassium phosphate buffer (pH 6.8). The cells were then pelleted into 2% (w/v) agar at 37 °C and 1 mm³ pieces were dehydrated through an ethanol series (30–100%, v/v) prior to infiltration with Spurr's low-viscosity resin (Timm & Steinbuechel, 1990). After polymerization, 60–80 nm sections were cut on a Reichert Ultracut OMU4 Ultramicrotome, collected on 300-square copper grids (Agar Scientific) and contrasted with uranyl acetate/Reynolds lead citrate (Timm & Steinbuechel, 1990). The sections were visualized at 80 kV using a Siemens 102 electron microscope and micrographs were taken on Agfa Scientia EM film (Agfa Gevaert). The percentage of the total cell volume occupied by the PHA granules (see Timm & Steinbuechel, 1990) was determined by analysis of the electron micrographs using an Apple Macintosh Graphics tablet.

Chemical analyses. Alginate was measured by the colorimetric method of Knutson & Jeanes (1968) after dialysing samples of culture supernatants overnight against water to remove any contaminating glucose; low-viscosity alginic acid (Sigma) was used as a standard. Glucose, gluconate and 2-ketogluconate concentrations in culture supernatants were determined by HPLC using an Aminex HPX 87H ion-exclusion column (Bio-Rad) maintained at 30 °C and linked to an Anachem-Gilson chromatograph operating at a flow rate of 0.4 ml min⁻¹ with 5 mM H₂SO₄ as the solvent. Eluents were analysed using a Bio-Rad RI monitor and/or a Dynamax UV detector linked to an Apple Macintosh computer.

Presentation of results. Where appropriate, results are ex-

pressed as the mean $\pm$ the SEM with the number of independent determinations in parentheses.

RESULTS

Strain selection

Attempts to obtain steady-state continuous cultures of *P. aeruginosa* mucoid strain M48 during growth under ammonia limitation (D 0.05 h⁻¹) with either glucose or glycerol as the carbon source were unsuccessful due to the very rapid loss of mucoidy exhibited by these cells. However, repeated subculturing of strain M48 under batch growth conditions in a glycerol-mineral salts medium resulted in the isolation of a slightly more stable mucoid strain which was designated M60. The latter was grown successfully in steady-state continuous culture under ammonia limitation, but still started to lose mucoidy 1–2 d after attaining steady-state, albeit at a slower rate than strain M48. *P. aeruginosa* M60 was therefore used in all subsequent experiments.

Fig. 1. Changes in mucoidy, alginate concentration and various enzyme activities during the growth of *P. aeruginosa* M60 in ammonia-limited continuous culture (D 0.05 h⁻¹). The results are from a single experiment but are typical of those obtained in five independent experiments (variation up to $\pm 10\%$ between experiments, except for glucose dehydrogenase which varied by up to $\pm 30\%$) and are expressed as a percentage of the values determined on day 0. (a) ●, Mucoidy; ○, alginate. (b) ●, GMD; ○, PMM; △, glucose dehydrogenase.

Fig. 2. Changes in the concentrations of glucose and extracellular products during the growth of *P. aeruginosa* M60 in ammonia-limited continuous culture (D 0.05 h⁻¹). The results are from the experiment shown in Fig. 1 and are typical of those obtained in five independent experiments (variation up to $\pm 30\%$ between experiments, except for alginate which varied by up to $\pm 10\%$). ●, Glucose; ○, 2-ketogluconate; □, gluconate; ▲, alginate.

Mucoidy and alginate production

The growth of *P. aeruginosa* M60 in ammonia-limited continuous culture (D 0.05 h⁻¹; 30 °C) with glucose or glycerol as the carbon source produced alginate concentrations of 1.56 and 1.63 g l⁻¹, respectively, in the culture supernatant during the initial steady-state growth period. These concentrations were equivalent to alginate production rates (q_{alginate}) of 0.097 g alginate h⁻¹ (g dry wt cells)⁻¹ [$= 8.3$ nmol min⁻¹ (mg dry wt cells)⁻¹] and 0.108 g alginate h⁻¹ (g dry wt cells)⁻¹ [$= 9.2$ nmol min⁻¹ (mg dry wt cells)⁻¹], respectively, indicating that alginate

was produced at similar rates from glucose and glycerol by *P. aeruginosa* M60.

The more-prolonged growth of *P. aeruginosa* M60 in ammonia-limited continuous culture (D 0.05 h⁻¹; 30 °C) with glucose as the carbon source led to a very substantial decrease in the percentage mucoidy of the culture within approximately 10 d of exiting the initial steady-state, and was accompanied by an essentially parallel decrease in the concentration of alginate in the culture supernatant (Fig. 1a). The capacity of washed cells to produce alginate from glucose *in vitro* also decreased in parallel with the loss of mucoidy (data not shown; see also below). In contrast, the culture density remained essentially unchanged over the entire experiment.

Plate measurements of percentage mucoidy showed that the loss of mucoidy was an all-or-nothing phenomenon (i.e. a given cell was either mucoid or non-mucoid), indicating that loss of mucoidy (like the original acquisition of mucoidy) was probably due to a genetic switch. The decrease in alginate production thus reflected the decreasing proportion of mucoid to non-mucoid cells in the culture, rather than a gradual decline in the ability of all of the cells to make alginate. Furthermore, the culture exhibited no detectable alginate activity during the course of the experiment (as evidenced by the absence both of low- M_r alginate and of uronic acids from the culture supernatant), indicating that the loss of mucoidy did not result from an increased capacity to degrade alginate.

Enzyme activities

The activities of GMD and PMM (representative enzymes of the alginate biosynthetic pathway) also decreased approximately in parallel with the loss of mucoidy and alginate production (Fig. 1b). The initial PMM activity

Table 1. Concentrations of glucose and various products in a continuous culture of *P. aeruginosa* M60 before and after loss of mucoidy

P. aeruginosa M60 was grown in ammonia-limited continuous culture (D 0.05 h⁻¹) with glucose as the carbon source. Concentrations of glucose and various intracellular and extracellular products were assayed as described in Methods. Day 0 was the last day of the initial steady-state period. The results are from the experiment shown in Figs 1 and 2, and are typical of those obtained in five independent experiments (variation up to $\pm 30\%$ between experiments, except for alginate which varied by up to $\pm 10\%$). In all cases the net increase in total product concentration closely matched the decrease in glucose concentration.

Substrate or product	Substrate or product concentration (g l ⁻¹)		
	Day 0	Day 10	Change
Glucose	3.87	0.05	-3.82
Alginate	1.56	0.08	-1.48
Gluconate	0	0.16	+0.16
2-Ketogluconate	1.18	6.61	+5.43
PHA	0	0.05	+0.05
Total products	2.74	6.90	+4.16

was well in excess of that required to support the observed rate of alginate production by the mucoid culture [$32.6 \text{ cf. } 8.3 \text{ nmol min}^{-1} (\text{mg dry wt cells})^{-1}$]. In contrast, the initial activity of GMD, the major kinetic control point of the alginate biosynthetic pathway in this organism (Tatnell *et al.*, 1994), was substantially less than that required to support alginate production [$3.6 \text{ cf. } 8.3 \text{ nmol min}^{-1} (\text{mg dry wt cells})^{-1}$], indicating that the activity of this enzyme was probably underestimated (see also Narbad *et al.*, 1990). Both enzyme activities decreased by $\geq 80\%$ over the 10 d decay period and thus behaved in an essentially identical manner to mucoidy and alginate production. It was concluded, therefore, that the loss of mucoidy and alginate production was probably caused by decreases in the activities of the alginate biosynthetic enzymes.

In contrast, the activities of various enzymes involved in the initial stages of glucose catabolism via either the 'internal' pathway (glucokinase and glucose-6-phosphate dehydrogenase) or the 'external' pathway (glucose dehydrogenase, gluconate dehydrogenase and 2-ketogluconate kinase plus 2-ketogluconate-6-phosphate reductase) stayed relatively unchanged or increased over the course of the experiment. Thus the glucokinase, glucose-6-phosphate dehydrogenase, gluconate dehydrogenase and 2-ketogluconate kinase plus 2-ketogluconate-6-phosphate reductase activities remained relatively constant, whereas glucose dehydrogenase activity increased by up to eight-fold (Fig. 1b). The combined activities of the subsequent Entner-Doudoroff enzymes (6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase) remained essentially unchanged.

The measured activities of these enzymes, with the apparent exception of gluconate kinase and 2-ketogluconate kinase plus 2-ketogluconate-6-phosphate reductase, were all high enough (within experimental error) to support the observed rates of alginate production and glucose consumption by the initial mucoid culture {ranging from $29.7 \text{ nmol min}^{-1} (\text{mg dry wt cells})^{-1}$ for glucose dehydrogenase to $423.0 \text{ nmol min}^{-1} (\text{mg dry wt cells})^{-1}$ for gluconate dehydrogenase cf. $8.3 \text{ nmol min}^{-1} (\text{mg dry wt cells})^{-1}$ for alginate production and $35.2 \text{ nmol min}^{-1} (\text{mg dry wt cells})^{-1}$ [$= 0.380 \text{ g h}^{-1} (\text{g dry wt cells})^{-1}$; see below] for glucose consumption}. The addition of PQQ to the glucose dehydrogenase assay did not increase the activity of the enzyme, indicating that the relatively low initial activity was not due to depressed PQQ synthesis. No gluconate kinase activity was detected, although indirect evidence based on the observed ability of washed cells to produce alginate from gluconate indicated that this enzyme was present (see below).

Formation of alternative products to alginate

Initial steady-state cultures of *P. aeruginosa* M60 were characterized by the presence not only of relatively high concentrations of alginate (1.56 g l^{-1} ; see above) and unused glucose (3.87 g l^{-1}) in the effluent medium, but also of 2-ketogluconate (1.18 g l^{-1}) (Fig. 2; Table 1). In contrast, the effluent medium contained no detectable gluconate, and electron microscopy of washed cells

Fig. 3. Electron micrographs showing the appearance of intracellular granules during the growth of *P. aeruginosa* M60 in ammonia-limited continuous culture ($D \ 0.05 \text{ h}^{-1}$). (a) 2.5 d, population 90% mucoid; (b) 8 d, population 5% mucoid.

showed that they contained virtually no intracellular granules (Fig. 3a). However, the concentrations of both alginate and glucose in the effluent medium subsequently fell to 0.08 and 0.05 g l^{-1} , respectively, during the course of the experiment, whereas the concentration of gluconate increased slightly to 0.16 g l^{-1} and the concentration of 2-ketogluconate increased severalfold to 6.61 g l^{-1} . In addition, the turbidity of sonicated cells increased significantly and a low-density precipitate became evident following the centrifugation of broken cells to produce a high-speed membrane fraction; electron microscopy of washed cells revealed the appearance of increased numbers of intracellular granules (Fig. 3b) which were very similar to those seen during PHA accumulation in ammonia-starved cultures of *P. aeruginosa* (Timm & Steinbuchel, 1990). These intracellular granules increased from $< 0.1\%$ of the cell volume at the start of the experiment to 8.9% at the end of the experiment, i.e. from a concentration of approximately 0 to 0.05 g l^{-1} , confirming

Table 2. Production of alginate by washed cells of *P. aeruginosa* M60 prepared from an initial steady-state continuous culture

Washed cells of *P. aeruginosa* M60 were prepared from an ammonia-limited continuous culture (D 0.05 h⁻¹) grown for up to 3 d under steady-state conditions with glucose as the carbon source. The rate of alginate production by washed cells incubated with various substrates was determined as described in Methods. The results are expressed as the mean $\pm$ SEM with the number of independent determinations in parentheses.

Substrate	Rate of alginate production (q_{alginate}) [g alginate h ⁻¹ (g dry wt cells) ⁻¹]
Endogenous	0.026 $\pm$ 0.003 (4)
Glucose	0.097 $\pm$ 0.021 (14)
Gluconate	0.094 $\pm$ 0.013 (10)
2-Ketogluconate	0.034 $\pm$ 0.005 (9)
Succinate	0.052 $\pm$ 0.008 (3)
Glucose + gluconate	0.087 $\pm$ 0.013 (8)
Glucose + 2-ketogluconate	0.061 $\pm$ 0.008 (5)
Gluconate + 2-ketogluconate	0.071 $\pm$ 0.016 (5)
Glucose + succinate	0.067 $\pm$ 0.015 (3)

that substantially more PHA was made by non-mucoid cells than by mucoid cells (see also Timm & Steinbuchel, 1990, 1992). Overall, therefore, the decrease in the concentration of glucose in the effluent medium during the loss of mucoidy (Δ glucose: -3.80 g l⁻¹) was closely matched by the *net* change in the total concentration of the products (alginate, gluconate, 2-ketogluconate and PHA; Δ products: $+4.16$ g l⁻¹).

These changes in the concentrations of glucose and its various intracellular and extracellular products during the course of the experiment were obviously reflective of changes in the rates of glucose utilization and product formation by the culture (although it should be noted that the calculated rates are only approximations since the culture, by definition, was growing under non-steady-state conditions once the changes had started). The increase in q_{glucose} [0.39 g h⁻¹ (g dry wt cells)⁻¹] was almost completely matched [0.37 g h⁻¹ (g dry wt cells)⁻¹] by the combined increases in $q_{\text{gluconate}}$ [0.01 g h⁻¹ (g dry wt cells)⁻¹], $q_{\text{2-ketogluconate}}$ [0.44 g h⁻¹ (g dry wt cells)⁻¹] and q_{PHA} [<0.01 g h⁻¹ (g dry wt cells)⁻¹] minus the decrease in q_{alginate} [0.09 g h⁻¹ (g dry wt cells)⁻¹], indicating that little or none of the extra glucose consumed by the non-mucoid cells was being metabolized to carbon dioxide.

It was concluded, therefore, that the decreased production of alginate which occurred as *P. aeruginosa* M60 became non-mucoid was only partially compensated by the combined production of an alternative biopolymer (PHA) and an extracellular metabolite (gluconate), and was more than compensated by the substantial production (presumably via gluconate) of a second extracellular metabolite, 2-ketogluconate. The increased production of the latter was therefore mainly responsible for the overall increase in glucose utilization by the non-mucoid culture. The pattern of gluconate and 2-ketogluconate production

over the course of the experiment was completely commensurate with the initially low (but subsequently increasing) glucose dehydrogenase activity and the initially high (and essentially unchanging) gluconate dehydrogenase activity.

The inability to measure [¹⁴C]glucose transport into mucoid washed cells of *P. aeruginosa* M60, together with the unavailability of [¹⁴C]gluconate and [¹⁴C]2-ketogluconate, precluded the use of transport studies to investigate further the relative contribution of the 'internal' and 'external' pathways of glucose metabolism during growth under conditions of alginate synthesis.

An alternative approach was therefore adopted in which the rate of alginate production from various substrates was measured *in vitro* using mucoid washed cells prepared from ammonia-limited continuous cultures (D 0.05 h⁻¹). The rates of alginate production from glucose and gluconate by these washed cells were not only very similar from each substrate [q_{alginate} 0.097 and 0.094 g alginate h⁻¹ (g dry wt cells)⁻¹, respectively], but almost identical to the rate from glucose by ammonia-limited continuous cultures [0.097 g alginate h⁻¹ (g dry wt cells)⁻¹]. In contrast, the rate from 2-ketogluconate was only slightly higher than the endogenous rate (Table 2). It was concluded, therefore, that the rate of 2-ketogluconate uptake and metabolism is very slow compared with that of glucose or gluconate, and that glucose either enters the cell unchanged or, more likely (in view of the presence of glucose dehydrogenase; see also Whiting *et al.*, 1976b), is oxidized by glucose dehydrogenase and enters the cell as gluconate.

Several other substrates (including glycerol, fructose and succinate) which are not involved in the early stages of glucose metabolism were also examined for their ability to support alginate synthesis by washed cells grown on glucose. Only succinate yielded a rate of alginate pro-

duction which was significantly above the endogenous rate, but this was still well below the rate from glucose or gluconate. The rates of alginate synthesis from various combinations of glucose, gluconate and 2-ketogluconate, and also from a mixture of glucose and succinate, were no higher (and in some cases were significantly lower) than from either glucose or gluconate alone. It was concluded, therefore, that the major kinetic control point for alginate synthesis is beyond the point(s) at which the pathways of glucose/gluconate and succinate metabolism converge, thus adding further credence to the view that the rate of alginate synthesis is mainly determined by the activities of one or more of the biosynthetic enzymes (Tatnell *et al.*, 1994).

DISCUSSION

The work described in this paper shows that the rapid and progressive loss of alginate production by *P. aeruginosa* strain M60 was accompanied by enzymic changes which led to the production of alternative products. Predominant amongst these changes were decreases in the activities of the alginate biosynthetic enzymes (represented by GMD and PMM) and an increase in the activity of the first enzyme in the pathway for the 'external' catabolism of glucose (glucose dehydrogenase), which were accompanied by the increased production of PHA, gluconate and 2-ketogluconate. The essentially parallel decreases in the percentage mucoidy, the rate of alginate production, and the activities of GMD and PMM indicated that these enzymes were essential for alginate production, and were consistent with the recent report that GMD is a major kinetic-control-point in the alginate biosynthetic pathway (Tatnell *et al.*, 1994).

It is now well established that conversion of non-mucoid *P. aeruginosa* to mucoidy is stimulated by a wide range of environmental signals; these include energy-limited conditions even though the production of alginate is a significantly energy-dependent process which can thus impose a severe drain on the energy economy of the cell (Terry *et al.*, 1992). Mucoid cells apparently attempt to minimize this energy drain by channelling carbon substrates as efficiently as possible into central energy-conserving pathways. This is exemplified by the recent report that the two enzymes responsible for the overall production of ATP (initially GTP) by substrate-level phosphorylation during the operation of the tricarboxylic acid cycle in this organism (succinyl-CoA synthetase and nucleoside diphosphate kinase) are down-regulated in AlgR2 mutants that are unable to produce alginate (Schlichtman *et al.*, 1994), thus implying that tricarboxylic acid cycle activity is higher in mucoid cells than in non-mucoid cells.

The observation that mucoid cultures of *P. aeruginosa* M60 excrete 2-ketogluconate when grown under ammonia limitation could suggest that, even when succinyl-CoA synthetase and nucleoside diphosphate kinase are operating at maximum capacity, the activity of the tricarboxylic acid cycle is insufficient to conserve energy at the rate required to support the observed rate of alginate

production [$0.097 \text{ g alginate h}^{-1} (\text{g dry wt cells})^{-1}$], and that the deficit is made up by additional energy conservation arising from oxidation of glucose to 2-ketogluconate by the 'external' pathway (see Hardy, 1992). However, this suggestion is incompatible with the observation that 2-ketogluconate is produced at much higher rates by non-mucoid cultures grown under the same conditions, particularly as the rate of carbon dioxide production (an indirect measure of tricarboxylic acid cycle activity) remains essentially unchanged during the loss of mucoidy.

A more satisfactory explanation is that ammonia-limited mucoid cultures are unable to regulate fully the rate at which glucose and/or its 'external' pathway metabolites are taken up by the cell (for other examples see Cornish *et al.*, 1988; Williams *et al.*, 1990). They consequently synthesize and export copious amounts of alginate both to overcome the potentially deleterious osmotic effects of accumulating surplus intracellular metabolites and to consume the surplus ATP produced by the further oxidation of these metabolites (see Linton & Rye, 1989). As the ability of the mucoid cells to make alginate subsequently decays due to the decreasing activities of the alginate biosynthetic enzymes, the ability of cells to consume surplus intracellular carbon and hence to turn-over excess ATP becomes greatly diminished. The nascent mucoid cells appear to overcome this problem in two ways. Firstly, by producing PHA, which is synthesized by a similarly energy-demanding pathway to alginate but which is osmotically inactive and hence remains intracellular; it should be noted, however, that the rate at which PHA is made is very slow compared with the rate of alginate production by the original mucoid cells [$0.010 \text{ cf. } 0.097 \text{ g h}^{-1} (\text{g dry wt cells})^{-1}$]. Secondly, and more importantly, by substantially increasing the activity of the slowest enzyme of the 'external' pathway of glucose metabolism (glucose dehydrogenase) without concomitantly increasing the ability of the cell to transport and metabolize 2-ketogluconate; this effectively draws glucose into the low-ATP-yielding and, under these conditions, largely dead-end external pathway, thus spilling surplus carbon as an extracellular metabolite and hence diminishing the production of excess ATP.

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