Control of Colanic Acid Synthesis

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Received for publication 10 April 1970

The nucleotide pools of certain mucoid, colanic acid-synthesizing strains of Escherichia coli, Salmonella typhimurium, and Aerobacter cloacae were examined, and in all cases the nucleotide sugars uridine-5'-diphosphate glucose (UDPG), uridine-5'-diphosphate galactose (UDPGal), guanosine-5'-diphosphate fucose (GDPF), and uridine-5'-diphosphate glucuronic acid (UDPGA) were detected. It is postulated that these nucleotide sugars are precursors in the synthesis of colanic acid. The levels of these nucleotide sugars and of the enzymes involved in their synthesis were examined in a number of mucoid strains and compared with the levels found in certain strains which were repressed in the synthesis of colanic acid, only becoming mucoid when grown in the presence of p-fluorophenylalanine (PFA). The levels of UDPG and UDPGal and the enzymes involved in their synthesis were substantially the same in both mucoid and repressed types, but the levels of UDPGA and GDPF and of some of the enzymes involved in their synthesis were much higher in mucoid strains. When repressed strains were grown in the presence of PFA, the levels of UDPGA and GDPF approached those found in mucoid strains. The existence of an operon, containing genes coding for certain key enzymes involved in colanic acid synthesis has been suggested.

The concept of an exopolysaccharide common to a large number of the Enterobacteriaceae has been discussed several times (1, 2, 10, 13) largely on the basis of the monosaccharide composition of various preparations examined. This common exopolysaccharide has been suggested to be colanic acid (27), originally isolated and characterized as an extracellular heteropolysaccharide from Escherichia coli K235, containing glucose, galactose, fucose, and glucuronic acid as its monomer constituents (7). The structure of this heteropolysaccharide has since been elucidated (14, 32), and a repeating hexasaccharide containing glucose, galactose, fucose, and glucuronic acid (1:2:2:1) has been postulated. In addition, the repeating unit contained a pyruvate residue attached in a ketal linkage to one of the galactose moieties and an o-acetyl group attached to the glucose moiety.

Markovitz (15–17, 19) has investigated the synthesis of colanic acid in E. coli K-12 and has shown that exopolysaccharide synthesis is controlled by at least two regulator genes. The product of one regulator gene, R1 (15), later designated Cap R (16), appeared to control several enzymes believed to be involved in colanic acid synthesis such as phosphomannose isomerase, uridine-5'-diphosphate galactose (UDPGal)-4-epimerase, and guanosine-5'-diphosphate fucose (GDPF) synthetase. Cap R was subsequently found in Shigella dysenteriae (18). Another regulator gene, R2 or Cap S (17), did not appear to control the level of any of the enzymes tested. Later work by Kang and Markovitz (12) indicated that many strains normally considered to be nonmucoid could be induced to become mucoid when grown in the presence of p-fluorophenylalanine (PFA). The exopolysaccharide produced under those conditions had the same chemotype as colanic acid, and a concomitant increase in UDPGal-4-epimerase and GDPF synthetase could be demonstrated. It was supposed that growing the cells in the presence of PFA interfered with the product of the regulator gene. The inference was that all strains of E. coli K-12 possessed the genetic ability for the synthesis of colanic acid, but that the majority had this ability repressed by a regulator gene. Strains which were normally mucoid were considered to be derepressed.

Recently, a more comprehensive study (8) of the occurrence of colanic acid within the Enterobacteriaceae has been made. A method of identifying colanic acid by "typing" it with certain bacteriophage-induced depolymerase enzymes was developed, and, on the basis of this and...
conventional chromatographic studies, the earlier inferences about the wide general occurrence of colanic acid were confirmed. It was suggested that colanic acid is a common antigen associated with the Escherichia, Salmonella, and Aerobacter groups rather than with all the Enterobacteriaceae. In addition, it was shown that many Salmonella strains considered nonmucoid could synthesize colanic acid when grown in the presence of PFA, suggesting that a similar pattern of repression and derepression exists within the Salmonellae as with E. coli K-12.

The results of Kang and Markovitz (12) and Markovitz (15) indicated that the control of synthesis, although genetic in origin, may lie at the nucleotide sugar level. We examined the nucleotide pools of certain mucoid strains, attempting to detect possible precursors involved in the synthesis of colanic acid. To gain more insight into the control mechanism, we also compared the nucleotide pools and levels of possible precursors in certain mucoid strains with those of certain repressed strains, grown on normal media and derepressed by growth in the presence of PFA.

MATERIALS AND METHODS

**Bacterial strains.** The following strains have already been described (8): E. coli K-12 strains S53, S61, S22, S8, S56, CA10, and CA3; A. cloacae NCTC 5920; and Salmonella typhimurium SL 1098. CA10R is a galactose-positive revertant of CA10. S22M, a mutant of S22, is mucoid only in the presence of PFA.

**Media.** Nutrient broth and solid minimal medium containing PFA have already been described (8). The liquid minimal PFA medium used was similar to solid minimal PFA medium, except that it was supplemented with 8 × 10−5 PFA as described by Kang and Markovitz (12).

**Preparations of nucleotide extracts.** Cells were grown in nutrient broth for 18 hr or in liquid PFA medium for 4 to 5 days in 200-ml shake cultures in 500-ml Erlenmeyer flasks at 30°C. Centrifugation of the cells was carried out at 20,000 × g with a continuous-action rotor at 0°C. The resultant cell paste was suspended in a small volume of ice-cold 0.85% (w/v) saline and poured into 3 volumes of boiling 95% (v/v) ethanol. While extracting the mixture with stirring for 15 min, the ethanol-water mixture was maintained at boiling point. Cell debris was removed by centrifugation at 300 × g, and the extract was concentrated to small volume on a rotary evaporator at 20°C. The extract was deproteinized by shaking with an equal volume of chloroform for 10 to 15 min and by centrifuging at 40,000 × g; the aqueous layer was removed and kept at −20°C until used.

**Analysis of nucleotide pools.** Column chromatography of nucleotide extracts was carried out on cellulose on columns (Whatman ETI1 Ecteola; 25 by 1 cm or 45 by 1.5 cm) by a modification of the method described by Nilsson and Sjunnesson (22). A 1.0 M concentration of triethylammonium acetate was prepared by adjusting a solution of triethylamine in distilled water to pH 4.0 or 6.0 with glacial acetic acid. ET11 cellulose powder was suspended in 0.5 N NaOH under vacuum for 30 min and then washed on a sintered glass pad under suction with distilled water until the washings were neutral. The powder was then resuspended in 1.0 M triethylammonium acetate (pH 4.0) and again degassed for 30 min. Columns were packed under gravity and equilibrated with the same buffer for 24 hr at a flow rate of 25 ml/hr. The column was then washed with distilled water for 24 hr at the same flow rate, and the nucleotide extract was absorbed by washing it into the top of the column with a little distilled water. Material from 20 g (dry weight) of cells could be applied to the larger column and 5 g (dry weight) to the smaller. A buffer gradient system was set up, comprising a closed mixer volume of 500 ml of distilled water into which a reservoir syphoned. Effluent from the mixer vessel was pumped through the column at 25 ml/hr, and the column effluent was continuously monitored at 254 nm.

Initially, water was passed through to elute any uncharged ultraviolet (UV)-absorbing substances. A large peak of material was quickly eluted with distilled water. A second elution with the same buffer returned to zero, the distilled water in the reservoir was replaced with 0.5 M triethylammonium acetate (pH 6.0), and 10-ml fractions were collected. About 1,200 ml of buffer was required to elute all the UV-absorbing material. Peaks were collected and freeze-dried several times to remove the solvent. Further fractionation was carried out by paper electrophoresis on Whatman no. 1 or 3 MM paper in pyridine-acetic acid-water (5:2:43, v/v/v, pH 5.3; reference 34). Guide strips were stained for phosphorus, and phosphorus-containing areas were eluted. Fractions obtained by electrophoresis were subjected to ascending chromatography on thin-layer plates of 0.3-mm thickness prepared from MN 300 cellulose (Macherey, Nagel and Co., Duren, Germany) incorporating 2% (w/w) luminescent material (type H 93 green; Levy West Laboratories, Ltd., Harlow, England) in the following solvent A, saturated (NH4)2SO4-1.0 M sodium acetate (pH 7.5)-isopropanol (80:18.2, v/v/v; reference 25) and solvent B, 0.1 M ammonium acetate-ethanol, (3:7, v/v; reference 24).

Nucleotides were identified by comparison with standards in electrophoresis and in solvents A and B. Nucleotide sugars were identified, in addition, by elution, hydrolysis in 0.01 N HCl at 100°C for 10 min, followed by chromatography in solvents A and B to identify the nucleotide diphosphate moiety released, and then by chromatography in solvent C (pyridine-butanol-water: 4:6:3, v/v/v; reference 37) and solvent D (pyridine-ethyl acetate-acetic acid-water: 5:5:1:3, v/v/v; reference 5) to identify the sugar moiety released.

UV-absorbing material was observed on thin-layer plates under illumination at 253.7 nm as dark-blue spots on a fluorescent-green background. Less than 1 µg could be detected. Sugars were observed in solvents C and D by the alkaline silver nitrate method of Trevelyan et al. (35).

Sugar nucleotides eluted from Ecteola columns...
were assayed as follows: uridine-5'-diphosphate glucuronic acid (UDPGA) was determined by the carbazole method (3); uridine-5'-diphosphate glucose (UDPG) was determined by using the enzymatic method of Strominger, Maxwell, and Kalckar (30); UDPGal was determined after hydrolysis in 0.01 N HCl at 100 C for 10 min, the galactose released being assayed by Galactostat reagent (Worthington Biochemical Corp.). GDPF was determined on material eluted after further fractionation by electrophoresis, by the method of Dische and Shettles (4).

Enzyme assays. Cell-free extracts were obtained by growing cells for 18 hr in nutrient broth and treating the cell suspension as follows: centrifuging at 20,000 X g, washing twice with ice-cold 0.85% (w/v) saline, suspending the cells from the 500-ml culture in 10 ml of ice-cold distilled water, disrupting them with ultrasonic disintegration at 20 kc/sec for 90 sec, and cooling in ice. The lysate was centrifuged at 100,000 X g for 30 min, and the supernatant fraction was kept on crushed ice. Assays were performed on freshly prepared supernatants.

UDPGal-4-epimerase was assayed by the two-step procedure of Imae, Morikawa, and Kurahashi (11); phosphoglucomutase was assayed by the method of Najjar (21); phosphoglucoisomerase and phosphomannose isomerase were assayed by the method of Slein (28); UDPG pyrophosphorylase was assayed by the method of Munch-Petersen (20); UDPG dehydrogenase was assayed by a modification of the method described by Strominger, Maxwell, and Kalckar (30). Since most of the UDPG dehydrogenase activity appeared to be bound to particulate material, broken-cell preparations were used, prepared in the same way as the cell-free extracts except that the final centrifugation at 100,000 X g was omitted. The assay mixture consisted of: 15 mm MgCl2; 150 mm glycine-NaOH (pH 8.7); 0.3 mm UDPG; and 0.5 μc/ml 14C-UDPG. After incubation at 30 C for 30 min, the reaction was stopped by boiling for 90 sec and then by transferring the mixture to crushed ice. Precipitated protein was removed by centrifugation at 1,000 X g, and the supernatant fraction was subjected to paper electrophoresis. The areas corresponding to UDPG and UDPGA were cut out and counted in a scintillator with a toluene base; GDPF synthetase complex was detected by using the reaction mixture described by Ginsburg (6). After incubation at 30 C for 1 hr, the reaction was stopped by boiling, and then by transferring the mixture to crushed ice. The precipitated protein was removed by centrifugation at 1,000 X g, and the supernatant was acidified to pH 1 with HCl. After heating at 100 C for 10 min, the mixture was desalted by electrophoresis in the previously described buffer, and the area of the origin was eluted and subjected to thin-layer chromatography in solvents C and D to determine the appearance of fucose.

Enzymes and chemicals. All the common bases, nucleotides, and nucleoside mono-, di-, and triphosphates were available commercially, as were UDPG, UDPGal, uridine-5'-diphosphate (UDP)-N-acetylglucosamine, guanosine-5'-diphosphate mannose (GDPmannose), UDPGA, and 14C-UDPG (76 mc/mmc); N-acetyluramidic acid was prepared by acetylyating commercially available muramic acid (31) and purified by electrophoresis; 2-keto, 3-deoxyoctonic acid (KDO) was prepared from a lipopolysaccharide (LPS). The following enzymes used in the assays were obtained commercially: glucose-6-phosphate dehydrogenase, phosphoglucomutase, phosphoglucose isomerase, and UDPG dehydrogenase.

RESULTS

E. coli S53, a strain which was mucoid on all types of media, was selected as a typical example of a colanic acid-synthesizing strain to establish which nucleotide sugars might be precursors in colanic acid synthesis. Nucleotide extracts were prepared and analyzed by a combination of column chromatography, paper electrophoresis, and thin-layer chromatography. A typical UV trace of the eluate from column chromatography of such an extract is shown in Fig. 1. In addition to the large peak of UV-absorbing material which was eluted from the column with distilled water, there were some eight to nine other major peaks which were eluted by the buffer gradient system. In this investigation we were particularly interested in those peaks containing sugars and did not identify many of the UV-absorbing substances which were detected on thin-layer chromatography, but released no sugar components on hydrolysis. The nucleotide sugars were eluted in three main peaks, peaks 4, 7, and 8. Of the other peaks, peak 2 was of interest because,
in addition to a mixture of oxidized and reduced coenzymes, it contained a small amount of a free sugar which proved chromatographically and electrophoretically identical to a sample of KDO. Since KDO is found in the LPS of E. coli K-12 (Grant, unpublished data), then presumably the free KDO is a breakdown product of cytidine-5'-monophosphate-KDO, the precursor involved in KDO transfer to LPS in E. coli (9). Peaks 5 and 6, in addition to being a mixture of UV-absorbing substances, also contained a mixture of mannose and glucose phosphates, identified on the basis of electrophoretic mobility and chromatography of the sugar released after hydrolysis in 1 N H2SO4 for 1 hr.

Of the three peaks containing sugar nucleotides, peak 4 contained a mixture of UDPG, UDPGal, GDPF, and thymidine-5'-diphosphate rhamnose (dTDPRh). Presumably dTDPRh is a precursor in LPS biosynthesis, since this strain, in addition to KDO, has heptose, glucose, galactose, rhamnose and N-acetylglucosamine in the LPS (Grant, unpublished data). Of the other three nucleotide sugars in this peak, two of the sugar moieties, glucose and galactose, are found both in LPS and colanic acid; the other, fucose, only in colanic acid. Peak 7 was a useful marker peak, being yellow from the presence of a flavin-containing coenzyme. This was probably flavin adenine dinucleotide, since hydrolysis released a UV-absorbing, nonfluorescent substance with the chromatographic mobility of adenosine diphosphate (ADP). The peak also contained a component which was chromatographically homogeneous in solvents A and B. When hydrolyzed, it gave a single UV-absorbing component identified chromatographically as UDP, and two different sugar components which gave positive tests for N-acetylated amino sugars. They were identified chromatographically as N-acetylglucosamine and N-acetylmuramic acid in solvents C and D. Presumably, the initial chromatographically homogeneous component was a mixture of UDP, N-acetylglucosamine and UDP-N-acetylmuramic acid, the first concerned in peptidoglycan biosynthesis, the second both in peptidoglycan and LPS biosynthesis. Peak 8 was chromatographically homogeneous and contained UDPGA, glucuronic acid being found only in colanic acid. No other nucleotide sugars were detected in the soluble pool, despite the fact that GDPF has been shown to be synthesized via GDPmannose in other systems, and that the heptose in the LPS and glucose in bacterial glycogen may have GDP- and ADP-linked precursors, respectively.

Similar analyses were made on the other mucoid K-12 strains and on *A. cloacae* and *S. typhimurium*. Although there were variations in the general pattern from strain to strain, in all cases the nucleotide sugars dTDPRh, UDPG, UDPGal, GDPF, and UDPGA were detected, and no other nucleotide derivatives of the five sugars were present. Also, *S. typhimurium* had a large amount of a cytidine-5'-diphosphate (CDP) sugar in the peak 4 region, which was probably CDP aquebous, a LPS precursor (36).

Quantitative analysis of the nucleotide derivatives of the four sugars found in colanic acid were made (Table 1), comparing the strains which were mucoid on all types of media with those which were only mucoid in the presence of PFA or were known mutants. Strains S56 and S22M were mucoid only in the presence of PFA, S22M having originally been isolated as a nonmucoid colony on a nutrient agar streak plate of S22, made from an old stock culture kept on nutrient agar. Analysis of the nucleotide pools of both strains (Table 1) grown in nutrient broth showed that the level of UDPGA was only about 5% of that found in mucoid strains, and that GDPF could no longer be detected chromatographically. UDPG and UDPGal were present at essentially the same concentration as in mucoid strains. However, when grown on PFA, the levels of UDPGA and GDPF approached those of normally mucoid strains. In strain CA3, a UDPGal4-epimerase-less mutant, analysis of the nucleotide pool showed that UDPGal was lacking, but that the overall pattern was similar to that of S22M and S56 and that, in the presence of PFA, the levels of GDPF and UDPGA increased markedly although the strain did not synthesize colanic acid, presumably because of the enzyme defect and unavailability of UDPGal. Strain CA10 is a UDPG pyrophosphorylase-less mutant and was expected to lack UDPG and also UDPGal and UDPGA, since these are synthesized from UDPG. However, small amounts of UDPG, UDPGal, and UDPGA were detected (Table 1), suggesting that the enzyme defect was leaky or that revertants arose in the culture. The latter appeared to be the case, since revertants were readily picked up by streaking the strain on galactose containing medium and purifying galactose positive colonies. One such revertant, designated CA10R, proved to be nonmucoid on ordinary media, but mucoid in the presence of PFA. Comparison of the nucleotide pools of the parent strain and CA10R revealed that, whereas the parent had low levels of UDPG, UDPGal, and UDPGA, CA10R had normal levels of UDPG, UDPGal, and a low level of UDPGA, similar to that observed with S22M and S56. On growing both strains in the presence of PFA, the levels of GDPF and UDPGA increased.
Salmonella strains have exception of many strains, in was assay colanic acid was nonmucoid obtained enzymes strains, phosphomannose and difference assays not have strains on several pathways of the nucleotide biosynthesis, leading to the synthesis of the sugar nucleotides have been shown in various organisms, including E. coli, but all of the reactions have not yet been shown in a single strain. Enzyme assays of some of these enzymes were performed on several of the strains grown in nutrient broth (Table 2). The pathways leading to the synthesis of the sugar nucleotides have been shown in various organisms, including E. coli, but all of the reactions have not yet been shown in a single strain. Enzyme assays of some of these enzymes were performed on several of the strains grown in nutrient broth (Table 2). With the exception of the mutant strains CA3 and CA10, there was very little difference between strains mucoid under these conditions and those nonmucoid with respect to the enzymes leading up to the synthesis of UDPG and UDPGal. However, an obvious feature was that the level of UDPG dehydrogenase was some 5 to 10 times higher in mucoid strains, phosphomannose isomerase was 50% increased in some strains, and GDPF synthetase was only detectable in the mucoid strains with the assay method used, complementing the results obtained from the nucleotide analysis.

**DISCUSSION**

Previous work (8) has indicated that, with the exception of strains which have a defect in a gene coding for a portion of the synthesis, all K-12 strains, many other E. coli strains, and many Salmonella strains have the genetic ability to synthesize colanic acid. Most strains are normally nonmucoid or repressed (15, 17) but many of these can be derepressed by growth in the presence of PFA (12). In an attempt to learn more about the repression of synthesis, examination of the nucleotide pools of certain mucoid strains of K-12, S. typhimurium, and A. cloacae revealed that the only nucleotide derivatives of the monomer components of colanic acid were UDPG, UDPGal, UDPGA, and GDPF. That fucose and glucuronic acid are found only in colanic acid in all of the strains examined is a strong indication of the likely involvement of GDPF and UDPGA in colanic acid synthesis. All of the strains except A. cloacae have glucose and galactose as components of the LPS; thus, the presence of UDPG and UDPGal is expected since these have been shown many times to be precursors in glucose and galactose transfer to LPS. The absence of any other nucleotide deriva-

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**Table 1. Nucleotide levels of various strains**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mucoid</th>
<th>UDPG</th>
<th>UDPGal</th>
<th>UDPGA</th>
<th>GDPF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NB PFA</td>
<td>NB PFA</td>
<td>NB PFA</td>
<td>NB PFA</td>
<td>NB PFA</td>
</tr>
<tr>
<td>S53</td>
<td>0.148</td>
<td>0.082</td>
<td>1.63</td>
<td>0.500</td>
<td>0.050</td>
</tr>
<tr>
<td>S61</td>
<td>0.161</td>
<td>0.072</td>
<td>1.48</td>
<td>0.072</td>
<td>NT</td>
</tr>
<tr>
<td>S22</td>
<td>0.097</td>
<td>0.068</td>
<td>1.15</td>
<td>0.037</td>
<td>NT</td>
</tr>
<tr>
<td>S22M</td>
<td>0.100</td>
<td>0.072</td>
<td>0.052</td>
<td>0.68</td>
<td>0.059</td>
</tr>
<tr>
<td>S56</td>
<td>0.095</td>
<td>0.087</td>
<td>0.029</td>
<td>0.47</td>
<td>0.071</td>
</tr>
<tr>
<td>S8</td>
<td>0.097</td>
<td>0.068</td>
<td>1.01</td>
<td>0.052</td>
<td>NT</td>
</tr>
<tr>
<td>CA3</td>
<td>0.083</td>
<td>0.091</td>
<td>0.062</td>
<td>0.31</td>
<td>0.029</td>
</tr>
<tr>
<td>CA10</td>
<td>0.011</td>
<td>0.023</td>
<td>0.010</td>
<td>0.047</td>
<td>0.037</td>
</tr>
<tr>
<td>CA10R</td>
<td>0.083</td>
<td>0.059</td>
<td>0.041</td>
<td>0.80</td>
<td>0.041</td>
</tr>
<tr>
<td>Aerobacter cloacae</td>
<td>0.130</td>
<td>0.097</td>
<td>1.80</td>
<td>0.027</td>
<td>NT</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>0.158</td>
<td>0.082</td>
<td>1.10</td>
<td>0.062</td>
<td>NT</td>
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</table>

* Abbreviations: UDPG, uridine-5'-diphosphate glucose; UDPGal, uridine-5'-diphosphate galactose; UDPGA, uridine-5'-diphosphate glucuronic acid; GDPF, guanosine-5'-diphosphate fucose; NB, indicates cells grown in the presence of nutrient broth; PFA, indicates cells grown in the presence of p-fluorophenylalanine; +, indicates degree of mucoidness; -, not detectable; and NT, not tried. Values are expressed as micromoles of nucleotide per gram (dry weight) of cells.
A. coli, and P. aeruginosa. The study established that the synthesis of these sugars does not necessarily mean that LPS and colanic acid have the same glucose and galactose precursors; however, the fact that mucoid A. cloacae has no galactose in the LPS (33), yet had UDPGal in the soluble pool, is strong circumstantial evidence for the involvement of the glucose and galactose precursors in colanic acid synthesis. In addition, the mutant strains CA3 and CA10 (UDPGal-4-epimerase-less and UDPG pyrophosphorylase-less, respectively) were unable to synthesize colanic acid under any conditions, but a revertant CA10R would synthesize colanic acid under the correct conditions.

Comparison of the nucleotide pools of mucoid strains with those of certain nonmucoid repressed strains grown under normal conditions and in the presence of PFA has revealed certain differences between the levels of nucleotide sugars found in both. In particular, whereas there was no appreciable difference between the levels of UDPG and UDPGal in both types, mucoid strains contained large amounts of UDPGA, sometimes up to 20 times the level found in repressed strains grown under normal conditions. Also, GDPF was found in the pool of mucoid strains, whereas it was chromatographically undetectable in nonmucoids. It is possible that nonmucoid strains grown under normal conditions may have a low level of GDPF undetectable with the method used, but at least it is clear that mucoid cells have much higher levels. Examination of the enzymes involved in the synthesis of these sugar nucleotides has revealed essentially a complementary situation. The enzyme UDPG dehydrogenase was at a much higher level in mucoid strains, and two enzymes in the GDPF synthesis pathway, GDPF synthetase and phosphomannose isomerase, also showed increased activity. The enzymes leading up to the synthesis of UDPG and UDPGal were essentially the same in both types. Furthermore, when repressed strains were derepressed by growth in the presence of PFA, colanic acid was synthesized and the levels of UDPGA and GDPF in such strains markedly rose. The comparison of these sugar nucleotide levels and of the enzymes involved in their synthesis in both types of strain strongly support the pathway outlined in Fig. 2 as being operative in colanic acid synthesis. Since it has been shown that colanic acid contains acetate and pyruvate groups in addition to the four sugars (14, 32), the question arises as to when these groupings are inserted into the molecule. No o-acetylated or pyruvylated sugar nucleotides were isolated; this may, however, be a reflection of the lability of these groups, especially o-acetyl groups in particular which are extremely labile to hydrolysis. However, the evidence available from other systems (26) indicates that acetate at least is normally inserted at the polysaccharide level. Few reports have shown the presence of pyruvate in bacterial polysaccharides and none have shown when it is inserted, but it seems likely that addition to the molecule would occur at a late stage in the synthesis in a manner similar to the insertion of acetate.

Given the genetic potential for the synthesis of

### Table 2. Enzyme levels of various strains

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mucoid on ordinary media</th>
<th>Mucoid on PFA</th>
<th>PGI</th>
<th>PGM</th>
<th>UDPGPP</th>
<th>EPIM</th>
<th>UDPGD</th>
<th>PMI</th>
<th>GDPF SYN</th>
</tr>
</thead>
<tbody>
<tr>
<td>S53</td>
<td>++</td>
<td>++</td>
<td>13.3</td>
<td>5.1</td>
<td>0.92</td>
<td>0.40</td>
<td>0.82</td>
<td>15.1</td>
<td>+</td>
</tr>
<tr>
<td>S61</td>
<td>+</td>
<td>+</td>
<td>13.1</td>
<td>3.8</td>
<td>0.81</td>
<td>0.68</td>
<td>0.97</td>
<td>17.2</td>
<td>+</td>
</tr>
<tr>
<td>S22</td>
<td>+</td>
<td>+</td>
<td>15.7</td>
<td>4.9</td>
<td>0.97</td>
<td>0.52</td>
<td>0.81</td>
<td>15.3</td>
<td>+</td>
</tr>
<tr>
<td>S22M</td>
<td>-</td>
<td>-</td>
<td>9.8</td>
<td>2.8</td>
<td>0.81</td>
<td>0.38</td>
<td>0.051</td>
<td>10.2</td>
<td>-</td>
</tr>
<tr>
<td>S56</td>
<td>-</td>
<td>+</td>
<td>10.1</td>
<td>3.1</td>
<td>0.73</td>
<td>0.41</td>
<td>0.072</td>
<td>12.8</td>
<td>-</td>
</tr>
<tr>
<td>CA3</td>
<td>-</td>
<td>-</td>
<td>10.1</td>
<td>3.9</td>
<td>0.81</td>
<td>-</td>
<td>0.091</td>
<td>12.2</td>
<td>-</td>
</tr>
<tr>
<td>CA10</td>
<td>-</td>
<td>-</td>
<td>9.8</td>
<td>5.1</td>
<td>0.34</td>
<td>0.41</td>
<td>0.052</td>
<td>8.7</td>
<td>-</td>
</tr>
<tr>
<td>CA10R</td>
<td>+</td>
<td>+</td>
<td>12.2</td>
<td>4.1</td>
<td>0.74</td>
<td>0.42</td>
<td>0.047</td>
<td>9.7</td>
<td>-</td>
</tr>
<tr>
<td><em>Aerobacter cloacae</em></td>
<td>++</td>
<td>++</td>
<td>17.2</td>
<td>5.1</td>
<td>0.97</td>
<td>0.60</td>
<td>0.82</td>
<td>19.1</td>
<td>+</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>++</td>
<td>++</td>
<td>21.1</td>
<td>6.1</td>
<td>0.98</td>
<td>0.49</td>
<td>1.21</td>
<td>22.0</td>
<td>+</td>
</tr>
</tbody>
</table>

*PFA, p-fluorophenylalanine; PGI, phosphoglucone isomerase; PGM, phosphoglucosmutase; UDPGPP, uridine-5'-diphosphate glucose, pyrophosphorylase; EPIM, uridine-5'-diphosphate galactose-4-epimerase UDPGD, UDPG dehydrogenase; PMI, phosphomannose isomerase; GDPF SYN, guanosine-5'-diphosphate fucose synthetase complex; +, indicates degree of mucoidness in first two columns and chromatographic detection of fucose in assay system in last column; and −, not detectable. At least three separate cultures of each strain were grown and assayed for the enzymes described. The results represent the average value for each strain and are expressed as micromoles of substrate disappearing or product appearing per milligram of protein per hour.
colanic acid in both mucoid derepressed strains and nonmucoid repressed strains, the system then presents an interesting problem in control. The repeating unit proposed for colanic acid is considerably more complex than any thus far described in other bacterial exopolysaccharides, probably requiring eight separate transferases for its assembly (32). Apart from any considerations of the complexity of control acting at the transferase level in such a system, the data do not support such a hypothesis in view of the differences in the levels of the postulated sugar nucleotide intermediates between mucoid and nonmucoid cells. The fact that the enzymes involved in the synthesis of two of the nucleotides are at a lower level in nonmucoid cells suggests that feedback inhibition or degradation of sugar nucleotides is not taking place. The most likely explanation is that the control of colanic acid synthesis is mediated through the regulation of the levels of UDPGA and GDPF at a genetic level by controlling the activity of one or more of the enzymes involved in their synthesis. Since a simultaneous increase in UDPGA and GDPF is observed when nonmucoid repressed cells are grown in the presence of PFA, this suggests that control occurs via a single repressor. This complements the results of Markovitz (15) and Markovitz and Rosenbaum (17), who showed the synthesis of GDPF and, to a lesser extent, the synthesis of UDPGal was under genetic control. In this investigation, the difference in levels of UDPGal and UDPGal-4-epimerase in mucoid and nonmucoid cells were not found sufficiently significant to suggest an exactly comparable situation. Assuming that such a control mechanism exists, it is then possible that at least some of the structural genes which code for enzymes in the pathway are groupd and form an operon, in much the same way that eight or nine enzymes involved in the biosynthesis of the O-specific sugars in S. typhimurium map in the same region of the bacterial chromosome (23, 29). It is unlikely that the genes coding for the synthesis of UDPG and UDPGal would be in such an operon, since these compounds are concerned in other biosynthetic reactions. In S. typhimurium, the genes coding for the synthesis of UDPG and UDPGal map in a different region from those concerned with O-specific sugars (29), presumably because of the diverse functions of these compounds. The same applies to the gene which codes for the first enzyme involved in GDPF synthesis, phosphomannose isomerase, probably, reflecting the fermentative function of this enzyme. If such a colanic acid operon exists, it would probably comprise the genes coding for UDPG dehydrogenase and the enzymes, other than phosphomannose isomerase, involved in the synthesis of GDPF. This would fit well with the apparently simultaneous alteration in the levels of only GDPF and UDPGA when nonmucoid strains are derepressed by growth in the presence of PFA.

ADDITIONAL

While this paper was being considered for publication, independent papers by Lieberman and Markovitz (J. Bacteriol. 101:965–972) and Lieberman, Shaparis, and Markovitz (J. Bacteriol. 101:959–964) were published on essentially the same topic, in which they also discuss the possibilities of a colanic acid operon. They showed (J. Bacteriol. 101:959–964) that UDPGA could be found in much larger quantities in mucoid E. coli K-12 Cap R strains and that the enzyme UDPG dehydrogenase was greatly increased in such strains. Lieberman and Markovitz were also able to show that GDPM pyrophosphorylase was elevated in mucoid strains (J. Bacteriol. 101:965–972). The second type of mucoid mutation, Cap S, was shown for the first time to derepress GDPM pyrophosphorylase and two of the enzymes involved in the conversion of GDPM to GDPF (J. Bacteriol. 101:965–972). No data on the level of UDPGA or UDPG dehydrogenase in mucoid Cap S strains were reported. In view of these recent results, it seems likely that our mucoid strains are of the Cap S type, since the only enzymes which we found to be more elevated were those specifically involved in the synthesis of GDPF and UDPGA.

LITERATURE CITED


