Inhibition of lipopolysaccharide synthesis in Agrobacterium tumefaciens and Aeromonas salmonicida

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Lipopolysaccharide (LPS) synthesis was inhibited, new lipid A metabolites accumulated, and growth ceased, when the plant pathogen Agrobacterium tumefaciens and the fish pathogen Aeromonas salmonicida were treated with an antibacterial agent which specifically inhibits CTP:CMP-3-deoxy-manno-octulosonate cytidylyltransferase (CMP-KDO synthase). The new lipid A metabolites were purified by chromatography on DEAE-cellulose and chemically analysed. Metabolites isolated from both bacterial species contained glucosamine and phosphate in a 1:1 molar ratio, and 3-OH-C14:0 was the major fatty acid present (1 mol and 1.4 mol per mol glucosamine for A. tumefaciens and A. salmonicida, respectively). Inhibition of LPS synthesis by CMP-KDO synthase inhibitor had no effect on the initial kinetics of A. tumefaciens attachment to cultured carrot cells, but did inhibit cell aggregation normally induced by bacterial cellulose synthesis. Bacteria treated with inhibitor remained viable and able to synthesize protein at 15%, the rate of control cells, indicating that the lack of cellulose-induced aggregation was not due to the inability of bacteria to make protein, but rather the inability to respond normally to the bacterial–plant cell interaction.

Introduction

Lipopolysaccharide (LPS) is a major component of Gram-negative bacteria that serves important roles in outer membrane structure and function, including the interaction of pathogens with their hosts. LPS from most Gram-negative organisms contains 3-deoxy-d-manno-octulosonate (KDO) which serves as a link between lipid A and core oligosaccharide units. The pathway of KDO synthesis and incorporation into LPS is known (Ray et al., 1982) and specific inhibitors of one of the enzymic steps [CTP: CMP-3-deoxy-d-manno-octulosonate cytidylyltransferase (CMP-KDO synthase)] have been designed (Capobianco et al., 1987) and transformed into antibacterial agents (Goldman et al., 1987). These new antibacterial agents have proven useful in the investigation of the early steps in synthesis and assembly of LPS in various Gram-negative bacteria (Goldman et al., 1988a, b, 1990; Kadam et al., 1989).

LPS synthesis ceases following addition of this new class of antibacterial agents to growing bacteria, resulting in accumulation of precursors to LPS as well as other lipid-A-related metabolites (Goldman et al., 1988a, b; Kadam et al., 1989). Both the direct precursor to LPS and the lipid-A-related side-products are capable of translocation to the outer membrane (Goldman et al., 1989; Osborn et al., 1980) resulting in an outer-membrane surface with reduced density of LPS (Goldman et al., 1989). The level of porin protein in the outer membrane is also reduced by more than 50% (Goldman et al., 1990). These alterations of outer-membrane structure result in increased sensitivity to other antibacterial agents and host immunodefenses (Goldman et al., 1987). The investigations summarized above were limited to several species of enteric (Salmonella, Escherichia, Serratia, Providencia, Citrobacter, Enterobacter) and one species of non-enteric (Pseudomonas aeruginosa) Gram-negative bacteria. More recently we have turned our attention to other non-enteric pathogens of fish and plants (Goldman et al., 1990).

Agrobacterium tumefaciens is a non-enteric Gram-negative pathogen which causes crown gall tumour
disease in wounded dicotyledonous plants (Lippincott & Lippincott, 1975). The sequence of basic events leading to an established infection is summarized as follows: (i) attachment of bacteria to plant cells exposed at wound sites; (ii) bacterial synthesis of cellulose fibrils and formation of bacterial aggregates; (iii) transfer of plasmid DNA; (iv) integration of Ti DNA into the plant genome; (v) alteration of plant growth hormone production; and (vi) establishment of crown gall tumour disease wherein the bacteria derive nutrients from the altered plant system. Aeromonas salmonicida is a pathogen of salmonid fish, capable of causing severe disseminated disease (Klontz et al., 1966). In this report we have used specific inhibitors of CMP-KDO synthase to investigate the assembly of LPS in A. salmonicida and A. tumefaciens, and the effects of LPS inhibition in A. tumefaciens on the initial events in the interaction with plant cells.

Methods

Bacterial strains and growth conditions. A. tumefaciens strains A61 and A136 (biotype 1), 3R32 (biotype 2) and Ag63 (biotype 3) were obtained from M. Thomashow (Michigan State University, East Lansing), while strain A6 (also biotype 1) was from the laboratory collection (R. Goldman). A. salmonicida subsp. salmonicida strain ATCC 33658 was obtained from the American Type Culture Collection, Rockville, MD. Salmonella typhimurium LT2 strain RG111 was from the laboratory collection (R. Goldman). Bacteria were grown in MOPS defined medium (Neidhardt et al., 1974) at 30 °C using glucose as the carbon source. N-Acetyl-D-glucosamine was added to 0.5 mM concentration when bacteria were radiolabelled with N-acetyl-D-[1-3H]glucosamine (Amersham). Sodium acetate (1 mM) was added when cells were radiolabelled with [3H]acetate (20 μCi ml⁻¹, Amersham). Casamino acids (Difco) was added to 1 mg ml⁻¹ to support growth of A. salmonicida. Sensitivity to antibacterial agents was determined by microtitre broth dilution and measurement of zones of growth inhibition by disk diffusion (1 μmol test compound per disk) in soft agar overlays (Goldman et al., 1987).

Analysis of inhibitor effects on LPS synthesis. Methods for analysis of inhibitor effects on LPS synthesis were previously described (Goldman et al., 1987, 1988a, b; Kadam et al., 1989). The inhibitor used was compound IV in our previous nomenclature (Goldman et al., 1987) which is z-C(1,5-anhydro-7-amino-2,7-dideoxy-D-manno-heptopyranose)carboxylate in amide linkage to the carboxyl terminus of L-alanylalanine (US Patents 4613589 and 4615390). Briefly, antibacterial agent was added to exponentially growing cultures of bacteria in MOPS medium containing 0.5 mM N-acetyl-D-glucosamine, followed immediately by addition of N-acetyl-D-[1-3H]glucosamine to 4 μCi ml⁻¹, or [3H]acetate to 20 μCi ml⁻¹. At the indicated times, samples were harvested, washed twice with 0.01 M-HEPES/NaOH buffer, pH 7.2, and when indicated, delipidated by extraction with 95% ethanol, followed by acetone and then diethyl ether, and extracted several times with phenol/chloroform/petroleum ether (2:5:8). The extract was evaporated to the phenol phase, which was loaded on to a DEAE-cellulose column equilibrated with methanol containing 1% (w/v) acetic acid. The column was washed with equilibration buffer and eluted with a linear gradient of 0 to 1 M-ammonium acetate. Radioactive peaks of interest were pooled, and material was extracted into chloroform as described previously (Goldman et al., 1988a, b). A modification of the Elson–Morgan reaction (Levvy & McAllen, 1959; Reissig et al., 1955) was used to determine glucosamine content after hydrolysis in 4 M-HCl at 100 °C. Phosphate content was determined by the method of Ames (1966). The fatty acid content of fractions was determined by gas chromatography. Samples containing the internal standard heptadecanoic acid were converted to methyl esters with 1:0 ml methanolic HCl and extracted into chloroform (see above). The samples in chloroform were taken to dryness and resuspended in a small volume of chloroform prior to injection into a 6 ft (2 mm diameter) glass column packed with 3% (w/v) SE30 on Gas-Chrom Q (80/100 mesh, Supelco). Analysis was done isothermally at 190 °C on a Hewlett-Packard model 402 gas chromatograph equipped with a flame ionization detector and a Varian model 4270 integrator. Values were determined in triplicate and standard deviations are reported.

Measurement of protein synthesis. Bacteria were grown in MOPS medium at 30 °C containing 180 μM-L-phenylalanine and labelled by addition of [1-14C]-L-phenylalanine (Amersham, 513 mCi mmol⁻¹) to 1.7 μCi ml⁻¹. Radiolabelled phenylalanine was evaporated to dryness prior to use in order to remove ethanol. Duplicate culture samples (0.5 ml) were removed and further incorporation of label stopped by mixing with 0.5 ml cold 10% (w/v) TCA containing 100 μg ml⁻¹ unlabelled phenylalanine. Samples were collected on GF/F glass fibre filters (Whatman) and washed 3 times with 5 ml 5% (w/v) TCA containing 50 μg unlabelled phenylalanine ml⁻¹ followed by 5 ml cold 95% (w/v) ethanol. Radioactivity was measured by liquid scintillation counting.

Bacterial interaction with carrot cells. The interaction of bacteria with cultured carrot cells was previously described by Matthysse et al. (1978). Briefly, bacteria grown in MOPS were either tested directly (control) or following exposure to the LPS inhibitor for 3–4 h. Overnight. The fraction of added bacteria attached to carrot cells was previously described by Matthysse et al., 1988a,b). A modification of the Elson–Morgan reaction (Reissig et al., 1955) was used to determine glucosamine content after hydrolysis in 4 M-HCl at 100 °C. Phosphate content was determined by the method of Ames (1966). The fatty acid content of fractions was determined by gas chromatography. Samples containing the internal standard heptadecanoic acid were converted to methyl esters with 1:0 ml methanolic HCl and extracted into chloroform (see above). The samples in chloroform were taken to dryness and resuspended in a small volume of chloroform prior to injection into a 6 ft (2 mm diameter) glass column packed with 3% (w/v) SE30 on Gas-Chrom Q (80/100 mesh, Supelco). Analysis was done isothermally at 190 °C on a Hewlett-Packard model 402 gas chromatograph equipped with a flame ionization detector and a Varian model 4270 integrator. Values were determined in triplicate and standard deviations are reported.

Chemicals. Radiochemicals were obtained from Amersham. Methanolic HCl kits were from Alltech Associates. DEAE-cellulose was from Whatman. 3-OH-C14:0 for use as a standard was synthesized chemically (Pugh et al., 1966). Fatty acids (C14:0, C16:0, C17:0 and C18:1) and all other chemicals were from Sigma.
Results and Discussion

Sensitivity of A. tumefaciens and A. salmonicida strains to CMP-KDO synthase inhibitor.

A. tumefaciens biotype 1 strains grew well in MOPS defined medium with glucose as the carbon source ($\mu = 0.57$ at 30 °C). The MIC for compound IV, determined by broth dilution, was 10 µg ml⁻¹ and zones of growth inhibition (Goldman et al., 1987) were clearly observed by disk diffusion (18–35 mm). Strain A61 was also sensitive to tri-L-ornithine (14 mm zone of inhibition) while strain A136 appeared resistant (no zone detectable). The MIC for compound IV was 10 µg ml⁻¹ on S. typhimurium RG111, and this strain was also sensitive to tri-L-ornithine (22 mm zone of inhibition). A. tumefaciens biotype 2 and 3 strains grew poorly in MOPS glucose medium. Although they were sensitive to compound IV, they were not studied further. The A. tumefaciens biotype 1 strains were as sensitive to compound IV as most enteric Gram-negative bacteria previously tested (Goldman et al., 1987, 1988b; Kadam et al., 1989; unpublished data); however, they appeared less sensitive to tri-L-ornithine. Both tri-L-ornithine (Bark & Gilvarg 1974) and compound IV (Goldman et al., 1987) enter Gram-negative bacteria by way of the oligopeptide transport (Opp) system. Inhibitory action of compound IV requires cleavage by intracellular peptidase (Goldman et al., 1987), whereas tri-L-ornithine inhibits protein synthesis directly (Bark & Gilvarg 1974). A. tumefaciens strains appear to contain a functional Opp system and appropriate intracellular peptidase activity to release the CMP-KDO synthase inhibitor from the peptide carrier. Genetic defects inactivating the Opp system occur at high frequency in most Gram-negative enteric bacteria (Hiles et al., 1987, and unpublished data). In contrast, mutants resistant to compound IV were present at a frequency of less than 10⁻⁸ in cultures of A. tumefaciens biotype 1 strains, and growth remained inhibited during overnight incubation with compound IV (Fig. 1). A. salmonicida was sensitive to compound IV (23 mm inhibition zone) with an MIC in broth dilution of 50 µg ml⁻¹.

Mode of action of compound IV on A. tumefaciens biotype 1 strains and A. salmonicida

The antibacterial activity of compound IV is limited to Gram-negative bacteria, and inhibition of LPS synthesis at the level of CMP-KDO synthetase was its mode of action for several species of Gram-negative enteric bacteria (Capobianco et al., 1987; Goldman et al., 1987, 1988a, b; Kadam et al., 1989). Inhibition of LPS synthesis caused accumulation of both direct precursor to LPS as well as lipid-A-related byproducts, and halted growth. The following results show that the same mode of action is responsible for antibacterial activity on A. tumefaciens biotype 1 strains and A. salmonicida. Essentially identical results were obtained with the three A. tumefaciens biotype 1 strains examined; only data derived from strain A136 will be presented.

Growth of A. tumefaciens gradually ceased following addition of compound IV in excess of 10 µg ml⁻¹ (Fig. 1). Only labelled phospholipid was extracted by delipidation of control or drug-treated cells (data not shown). LPS and residual phospholipid were extracted, by acidic methanol, from control cells radiolabelled with N-acetyl-D-[^1-3H]glucosamine (Fig. 2a). In contrast, when cells were treated with compound IV, incorporation of label into LPS decreased and two new metabolites appeared that were labelled with N-acetyl-D-[1-3H]glucosamine (Fig. 2a). Quantitative analysis revealed that LPS synthesis was inhibited by more than 90%, and that the two new metabolites were undetectable in control cells ($<5\%$ of the amount present in treated cells). These new metabolites were also radiolabelled with acetate, recovered from the TLC, and the labelled lipid was converted to fatty acid methyl esters for analysis by argentation chromatography. This technique separates the saturated, unsaturated and hydroxy classes of fatty acid methyl esters. Hydroxy fatty acids represented $>93\%$ of the fatty acids released from the major and minor new metabolites that accumulated in biotype 1 strains. In contrast, the fatty acid methyl esters released from phospholipid contained
mainly saturated and unsaturated fatty acids (>96%), and only small amounts of hydroxy fatty acid were present (<4%). These data show that compound IV inhibits LPS synthesis in A. tumefaciens, resulting in the appearance of two new metabolites which are radiolabelled with glucosamine, and contain hydroxy fatty acids.

Similar results were obtained with A. salmonicida. Growth gradually ceased following addition of greater than 50 μg compound IV ml⁻¹, LPS synthesis was inhibited, and two new metabolites appeared (Fig. 2b). These new metabolites were also radiolabelled with acetate and recovered from the TLC. More than 94% of the fatty acids released from the two new metabolites were hydroxy fatty acids, while fatty acid released from phospholipid contained mainly saturated and unsaturated fatty acid (>93%).

**Purification and chemical analysis of accumulated lipid A metabolites**

Radiolabelled lipid A metabolites, accumulated following addition of compound IV to A. tumefaciens strain A136 and A. salmonicida, were extracted from 11 delipidated cells, and fractionated on DEAE-cellulose (Fig. 3a). Two peaks eluted from the column during the wash with equilibration buffer and four peaks were bound and eluted with a subsequent ammonium acetate gradient during analysis of material extracted from A. tumefaciens. Peaks 1–4 were collected for further analysis by TLC. These fractions contained the following
components by TLC analysis: (peak 1), material co-
migrating with LPS and phospholipid; (peak 2), phospho-
lipid; (peak 3), the faster-migrating metabolite, designat-
ed 2 in Fig. 2(a); and (peak 4), the slower-migrating meta-
bolite, designated peak 1 in Fig. 2(a). Peak 1 contained
13-9 nmol phosphate and less than 6 nmol glucosamine,
while peak 2 contained 490 nmol phosphate and less than
5 nmol glucosamine. The yield of phosphate, glucosa-
mine, and 3-OH-C14:0 from peak 3 was 74-0 (± 0-6), 85-0
(± 16-2) and 106-3 (± 3-5) nmol, respectively (n = 3).
Given the range in the glucosamine data, it appears that
these components were present in approximately equal
molar ratios. The low amounts of peak 4 precluded
complete analysis; however 27-9 (± 5-9) and 16-2 (± 1-7)
nmol 3-OH-C14:0 and C16:0, respectively, were
recovered. LPS from A. tumefaciens is not well character-
ized, but 3-OH-C14:0 and 3-OH-C16:0 were reported as
the major fatty acid constituents from strains 0362 and
TT111 (Salkinoja-Salonen & Boeck, 1978). Our analysis
of LPS (Fig. 4) and LPS precursor prepared from strain
A136 yielded 3OH-C14:0 as the major fatty acid
component. The fatty acid composition we determined
for LPS was 11 (± 0-1) μmol 3-OH-C14:0, 1-7 (± 0-3)
μmol C18:1 and 1-2 (± 0-1) μmol C16:0 per g. Since
3OH-C16:0 is not available as a standard to use for
determining retention time and response factor, we
cannot be certain as to its presence or absence in LPS and
LPS precursor from A. tumefaciens. It is possible that the
minor peak preceding the internal standard (C17:0) is
3OH-C16:0, or that 3OH-C16:0 is not separating from
C17:0 in our gas chromatographic system (Fig. 4).
Additional studies would be required to answer this
question.

Similarly, multiple peaks were recovered by chromato-
ography of the extract prepared from A. salmonicida on
DEAE-cellulose (Fig. 3b). Peak 4 contained both of the
metabolites detected by TLC (Fig. 2b) free from phospholipid and other metabolites, while phospholipid
was the major component of peaks 3a and 3b. Peaks
1 and 2 were not examined further. Material recov-
ered from peak 4 yielded (n = 3) phosphate
(116-8 ± 0-3 nmol), glucosamine (118-7 ± 8-7 nmol) and
3-OH-C14:0 (162-6 ± 8-3 nmol), demonstrating the
presence of one phosphate per glucosamine. The
relatively higher molar ratio for 3-OH-C14:0 (1:37 per
mol glucosamine) coupled with data accuracy, may
reflect the partial loss of ester-linked 3-OH-C14:0 during
extraction (i.e. the ratio may be 2 per mol glucosamine).
Our fatty acid analysis of LPS gave 61 (± 3-5) μmol 3-
OH-C14:0, 5-1 (± 0-2) μmol C16:1, 15-3 (± 1-2) μmol
C12:0 and 2-8 (± 0-2) μmol C14:0 per g. Glucosamine
content was not determined.

Although more than one lipid A metabolite accumu-
lates when the KDO pathway is inhibited in various
Gram-negative bacteria (Goldman et al., 1987, 1988a, b;
Kadam et al., 1989; Raetz et al., 1985), a single lipid A
metabolite, designated IVₐ, was identified as the direct
precursor to mature LPS (Goldman et al., 1988a; Kadam
et al., 1989). This same metabolite is also the preferred
substrate to which KDO is added in an in vitro system
(Brozek et al., 1989). Metabolite IVₐ was the major
component accumulated, and was the first to appear
following inhibition of LPS synthesis. Minor compo-
nants which were not direct precursors to LPS accumu-
lated gradually with time, and only after the major
component had reached near-maximum values (Gold-
man et al., 1988a). Similarly in A. tumefaciens the major
metabolite (TLC peak 2, Fig. 2a) appeared earlier than
the minor component (peak 1) and chased more
efficiently to LPS (data not shown). The slower
migrating species (peak 2, Fig. 2a) in A. tumefaciens also
contained C16:0, which is not a normal component of A.
tumefaciens LPS (Salkinoja-Salonen & Boeck, 1978). We
found less than one C16:0 for each

Fig. 4. Fatty acid analysis of LPS prepared from A. tumefaciens strain
136. LPS was purified, and samples containing the internal standard
heptadecanoic acid were converted to methyl esters with methanolic
HCl. Methyl esters were extracted into chloroform prior to injection
into the gas chromatograph.
the involvement of LPS, protein, and LPS-protein complexes as the bacterial components involved in the disease process to continue, and avirulent cells were observed for strain A6. Bacteria treated with drug for 24 h were still as able to attach to plant cells as control bacteria.

Attachment of *A. tumefaciens* to wound sites on plants involves a specific, but incompletely defined interaction between the bacterium and plant cell surfaces (see Matthysse, 1986 for review). This interaction is required for the disease process to continue, and avirulent strains did not inhibit bacterial attachment to potato tuber slices (Pueppke & Benny, 1984), and avirulent mutants with altered LPS were recently shown to attach normally to plant cells (Metts et al., 1991). Other studies have implicated bacterial surface proteins as components of the initial bacterial–plant cell interaction (Matthysse, 1987). Analysis of bacterial attachment is further complicated by the facts that (i) not all bacteria in a population of a given strain are able to bind (Matthysse, 1986), indicating that the attachment phenotype is not fully expressed in all bacteria under the incubation conditions used, and (ii) non-specific effects of LPS on bacterial attachment and subsequent events in tumour formation have not been ruled out.

Inasmuch as compound IV inhibits LPS synthesis in *A. tumefaciens*, we investigated the effects of such inhibition on the ability of *A. tumefaciens* to attach to plant suspension culture cells. Bacteria growing in MOPS medium were treated with 20 μg compound IV ml⁻¹ for 3–4 h and then tested for their ability to bind to plant suspension culture cells. Attachment of treated cells was identical to that of control cells during the initial 60 min (Fig. 5) demonstrating that inhibition of LPS synthesis had no effect on the initial kinetics of the attachment process. Binding at later time periods was not studied in detail, owing to complexities in the analysis of control samples (trapping and binding of bacteria to cellulose fibrils, and growth of bacteria upon recovery from the shift down into plant tissue culture media). Binding of drug-treated bacteria, which do not replicate, did plateau at a value of 30% bacteria bound at 60 and 120 min. Aggregation of plant cells by bacterial cellulose occurred as expected in control experiments (Matthysse et al., 1981), but bacterial aggregation induced by cellulose was inhibited upon incubation with 20 μg compound IV ml⁻¹ (data not shown). Similar results were observed for strain A6. Bacteria treated with drug for 24 h were still as able to attach to plant cells as control cells incubated for 24 h in MOPS alone (data not shown). Microscopic observation showed no differences between attachment of control or drug-treated cells to plant cells, and normal cytoplasmic streaming was maintained in plant cells.

Since compound IV was included at 20 μg ml⁻¹ during the incubation of bacteria with carrot cells, we examined the effect of prolonged exposure. Compound IV had no effect on viability of *A. tumefaciens* strain A136 even after 24 h incubation at 30°C (data not shown) even though growth ceased after 4–5 h. Radiolabeled phenylalanine was incorporated into growing bacteria at a rate of 1.7 × 10⁴ d.p.m. min⁻¹ (mg cell protein)⁻¹, and incorpor-
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