Bacteriophage-resistant Mutants of *Escherichia coli* K12 with Altered Lipopolysaccharide. Studies with Concanavalin A

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Three classes of mutants of *Escherichia coli* K12, isolated by selection for resistance to lipopolysaccharide-specific bacteriophages, were agglutinated by Concanavalin A which is presumed to interact with the lipopolysaccharide component of the outer membrane. Wheat germ and soy bean agglutinins did not agglutinate the parent or mutant strains. The adsorption of certain bacteriophages was also inhibited by Concanavalin A. The pattern of inhibition of adsorption of bacteriophages suggests that non-specific masking of receptors may occur, as well as specific masking of terminal glucose residues. Although bacteria were agglutinated by Concanavalin A, the permeability of the outer membrane seemed unaffected.

INTRODUCTION

Plant lectins are a group of proteins which interact in a highly specific way with polysaccharides (Sharon & Lis, 1972). Concanavalin A (Con A) is one such protein, isolated from the Jack Bean, which binds specifically to α -linked, terminal, non-reducing D-glucose, D-mannose or N-acetyl-D-glucosamine residues (Goldstein & Iyer, 1966). For precipitate formation with Con A, the polysaccharide must be branched (Goldstein, Hollerman & Merrick, 1965; Goldstein, Hollerman & Smith, 1965) and Con A is considered to interact with unmodified hydroxyl groups at the C-3, C-4 and C-6 positions of the pyranose ring. Wheat germ agglutinin is reported to have specificity for N-acetylglucosamine (Biddle, Cronin & Sanders, 1970) and soy bean agglutinin for D-galactose, although this is low relative to its specificity for β -D-galactosamine (Lis *et al.*, 1970). Lectins have been widely and successfully used for studying the cell surface of animal cells, where they interact with glycoproteins (Nicholson, 1972*a*; Lis & Sharon, 1973).

Recently, Con A has been applied to the study of the lipopolysaccharide (LPS) of Gram-negative bacteria. It has been shown to interact with multivalent polysaccharide preparations from LPS of *Salmonella*, *Shigella* and *Escherichia* using immunological methods (Goldstein & Staub, 1970; Mayer, 1972; Mayer, Schlecht & Gromska, 1975). All these polysaccharide preparations contain glucose in the O antigen moiety or the core region. Concanavalin A also interacts with the glucose or N-acetylglucosamine residues of the cell wall teichoic acid of certain Gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus* (Archibald & Coapes, 1972; Doyle & Birdsell, 1972; Birdsell & Doyle, 1973). In these bacteria Con A has two major effects: agglutination of cells and the inhibition of adsorption of certain bacteriophages which require glucose or N-acetylglucosamine residues as at least a part of their receptor.

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Since the outer core of the LPS of *Escherichia coli* K12 contains glucose (Boman & Monner, 1975; Benedetto, Bruneteau & Michel, 1976; Prehm *et al.*, 1976*b*; Picken & Beacham, 1977), mutants in which these residues are exposed should interact with Con A. In this paper we report studies on whole cell agglutination and phage adsorption that confirm this prediction. We have also attempted to determine whether or not interaction of Con A with the LPS causes any perturbation of the outer membrane by looking for a change in permeability.

METHODS

Bacterial strains. Full descriptions and sources of the *E. coli* K12 parent strain AB1157-W30, its mutant strains, strain K1060-18T1 and *Salmonella typhimurium* TV119 are given by Picken & Beacham (1977). The fatty-acid auxotroph K1060-2/UCB19 is a U3-resistant mutant of K1060-2: F⁻ *thi fabB fadE rpsL trp* (Beacham & Silbert, 1973).

Media and growth conditions. These were as described by Picken & Beacham (1977).

Cell agglutination by lectins. Washed bacterial suspensions in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer (10 mM, pH 7.2) were equilibrated at 37 °C and, at time zero, Con A (100 µg ml⁻¹), wheat germ agglutinin (100 µg ml⁻¹) or soy bean agglutinin (500 µg ml⁻¹) was added. Controls contained no lectin or, in experiments with Con A, 100 mM- α -methyl-D-glucoside (α -MG), a competitive inhibitor, in addition to Con A. Samples were taken at intervals and examined by phase contrast microscopy. Though some early experiments indicated that low concentrations of Ca²⁺ ions were necessary for agglutination (Picken & Beacham, 1975), later experiments have shown that they are not necessary. No agglutination occurred in the presence of α -MG. Lectins were obtained from Miles-Yeda, Rehovot, Israel, or Boehringer.

Bacteriophage adsorption. Bacteria were grown in R broth and harvested in the early-exponential phase (equivalent to 5×10^7 organisms ml⁻¹). They were washed twice with 10 mM-HEPES buffer pH 7.2 containing the adsorption requirements of the particular phage in use: for Br2 adsorption, 0.01 M-CaCl₂; for T4, 0.1 M-NaCl; and for T6, 0.1 M-NaCl and 100 µg tryptophan ml⁻¹. The bacteria were resuspended in the same buffer at a concentration of 2.5×10^8 organisms ml⁻¹ and Con A was added at a range of concentrations from 0 to 800 µg ml⁻¹ or, in the kinetic experiments, at 1 mg ml⁻¹. Controls contained 100 mM- α -MG. At time zero, phage was added at a multiplicity of infection of 1:2000. Samples were withdrawn at intervals, diluted 100-fold in buffer containing chloroform (Adams, 1959) and the titre of free phage was determined in duplicate. The adsorption constant, *K*, was calculated from the formula $(2.3/Br) \log_{10}(P_0/P_t)$ (see Adams, 1959). During these experiments the bacteria did not precipitate to the bottom of the tube, although they were observed microscopically to be agglutinated.

Enzyme release. Bacteria were grown in R broth, harvested in the early-exponential phase (5×10^7 organisms ml⁻¹), washed three times with 10 mM-HEPES buffer pH 7.2 containing 0.1 mM-CaCl₂, and resuspended in this buffer at a concentration of 1.25×10^8 organisms ml⁻¹. Bacterial suspensions were equilibrated at 37 °C and Con A was added at 1 mg ml⁻¹. Controls contained no Con A or Con A and 100 mM- α -MG. Samples (2 ml) were taken at 15 min intervals and centrifuged at 22 500 *g* for 15 min. The bacteria were resuspended in 2 ml buffer, and shaken with toluene. Both bacteria and supernatants were assayed for alkaline phosphatase using *p*-nitrophenyl phosphate as substrate (Schlesinger, 1967).

Gentian violet uptake. This was carried out as described by Gustafsson, Nordström & Normark (1973). Bacteria were pre-incubated with Con A at 1 mg ml⁻¹. Controls contained no Con A.

Actinomycin D uptake. Actinomycin D uptake studies and treatment with EDTA were carried out as described by Voll & Leive (1970). Bacteria were treated with Con A at 1 mg ml⁻¹ for 15 min at 37 °C before addition of [³H]actinomycin D (0.77 µCi ml⁻¹, 4 µg ml⁻¹).

RESULTS AND DISCUSSION

Cell agglutination

Since a uniform feature of the binding of lectins to cells is whole cell agglutination, we tested a variety of wild-type and mutant strains for agglutination (Table 1). Three classes of U3-resistant mutants were agglutinated by Con A, but the parent strain was not. This is in full accord with the presence in the LPS of *E. coli* K12 of three glucose residues (Benedetto *et al.*, 1976; Prehm *et al.*, 1976*b*; see also Picken & Beacham, 1977). The LPS analysis and phage type of these mutant classes were reported by Picken & Beacham (1977). It is

Fig. 1

Fig. 1. The inhibition by Con A of adsorption of bacteriophage Br2 to the *E. coli* K12 mutant strain U440. ■, Average result from eight experiments. ▲, ●, ○, Results from three different experiments.

Fig. 2

Fig. 2. The inhibition by Con A of adsorption of bacteriophage Br2 to *S. typhimurium* TV119. ▲, ●, Results from two different experiments.

significant that all three classes are sensitive to phage Br2 which optimally requires terminal glucose (or *N*-acetylglucosamine) residues as receptors (Wilkinson & Stocker, 1968; Lindberg, 1973).

Salmonella typhimurium, which has a terminal *N*-acetylglucosamine residue in its LPS core polysaccharide (Wilkinson & Stocker, 1968), was also agglutinated by Con A but not by wheat germ agglutinin. We presume that the latter has more precise structural requirements which are not fulfilled. None of our mutants were agglutinated by wheat germ or soy bean agglutinins.

The reason for the relatively weak agglutination of strain AB1157-W31 is unknown, as is the surprising observation that another strain, K1060-18T1, carrying the same *galE* allele is not agglutinated at all (Table 1). Presumably the glucose III (see Fig. 2, Picken & Beacham, 1977) residue is not exposed in a favourable manner. We have suggested that a very small number of LPS core chains of *E. coli* K12 terminate in galactose, attached to the glucose III residue. We presume that this galactose, together with the suggested unfavourable configuration of glucose III, results in the parent strain AB1157-W30 not being agglutinated.

The work of Mayer *et al.* (1975) suggested that phosphate groups linked to the heptose residues hinder interaction with Con A. This may account for the fact that mutants U2 and T4235, both of which we postulate to have a terminal glucose I residue like U1020, were not agglutinated; in addition to the proximity of any phosphate groups, the proximity of the

Fig. 3. The effect of Con A on the kinetics of bacteriophage Br2 adsorption to whole cells of *E. coli* K12 mutant strain U440. Δ , Cells pre-incubated with Con A for 15 min, to block all adsorption sites, before addition of Br2. $\blacktriangle$, Adsorption of Br2 to untreated cells. $\bullet$, Con A added to adsorption mixture at (a) 2 min or (b) 4 min. $\circ$, Con A added to adsorption mixture at (a) 2 min or (b) 4 min, and α -MG added 6 min later.

postulated heptose side-chain may cause steric hindrance, as it seems to in the case of phage attachment.

Bacteriophage adsorption

Phage Br2 requires terminal glucose (or *N*-acetylglucosamine) residues in the LPS for adsorption to *S. typhimurium* (Wilkinson & Stocker, 1968). The observation that strain U440 is both sensitive to Br2 and agglutinated by Con A is consistent with this conclusion. It would therefore be anticipated that adsorption of Br2 to U440 would be prevented by Con A, through masking of the required glucose residues. Our results (Fig. 1) demonstrate that Con A does block the adsorption of Br2 to U440, and increasing concentrations of Con A caused a corresponding decrease in the rate of adsorption. A Con A concentration of $700 \mu\text{g ml}^{-1}$ was sufficient to prevent adsorption completely. Figure 2 shows the results of similar experiments using the *S. typhimurium* strain TV119. Addition of $1 \text{ mg Con A ml}^{-1}$ at 2 min to an adsorption mixture of Br2 and U440 caused not only the cessation of adsorption but also desorption of all previously adsorbed phage (Fig. 3a); subsequent addition of α -MG caused resorption to occur. Addition of Con A at 4 min produced the same qualitative effect, except that not all the adsorbed phage were desorbed (Fig. 3b). We interpret this to

Table 1. *Agglutination by lectins*

All mutant classes are described in Picken & Beacham (1977), and are derived from AB1157-W30. Two to four strains from each class were tested as described in Methods. Agglutination of U440 and AB1157-W31 was apparent within 5 min and maximal within 20 min, whereas agglutination of U1020 usually took about three times as long. Strains of mutant classes 9 to 12, including T4235, were also not agglutinated by Con A.

Class	Strain	Lectin*		
		Con A	WGA	SBA
	TV119	+++	—	—
1	AB1157-W30	—	—	—
2	AB1157-W31	++	NT	NT
	K1060-18T1	—	—	—
3	U440	++++	—	—
4	U2	—	—	—
5	U1020	++++	—	—
6	U110	—	—	—
7	C35	—	NT	NT

+ to + + + +, Approximate indication of clump formation; —, no clump formation; NT, not tested.

* Con A, Concanavalin A; WGA, wheat germ agglutinin; SBA, soy bean agglutinin.

Table 2. *Adsorption rates of bacteriophages Br2, T4 and T6 to E. coli strain U440*

Results show the rate of adsorption as a percentage of the rate in the absence of Con A.

Phage	Adsorption rate with Con A at:	
	500 $\mu\text{g ml}^{-1}$	1 mg ml^{-1}
Br2	23.9	7.2
T4	89.5	15.8
T6	73.0	23.4

mean that between 2 and 4 min the adsorbed phages change from a state of reversible to irreversible adsorption.

The effects of Con A on the adsorption of phage T6, which requires an outer-membrane protein receptor for adsorption (Michael, 1968; Verkleij, Lugtenberg & Ververgaert, 1976; Manning & Reeves, 1976), and on phage T4, which requires terminal or non-terminal LPS glucose (Prehm *et al.*, 1976a; Picken & Beacham, 1977), are shown in Table 2. Con A inhibited the adsorption of T4 and T6 to U440 but to a lesser extent than phage Br2. The difference between the effect of Con A on Br2 and on T4 and T6 is particularly marked at 500 μg Con A ml^{-1} . Adsorption of phage T6 to AB1157-W30, which is not agglutinated by Con A, was only reduced to 61% by 1 mg Con A ml^{-1} (data not shown); the reduction may nevertheless indicate the presence of some glucose exposed at the surface of this parental strain which reacts with Con A, though insufficiently to cause cell agglutination.

The inhibition by Con A of the adsorption of phage T6 to strain U440, albeit to a lesser extent than Br2, indicates that some non-specific masking of receptors by Con A might occur. In other words, the binding of Con A to glucose residues prevents adsorption of a glucose-specific phage (Br2) and seems to render other receptors less available to certain phages, perhaps by steric hindrance. Phage T4 may also be only sterically hindered. But since this phage requires LPS glucose for its adsorption (Hattman & Fukasawa, 1963; Dawes, 1975; Prehm *et al.*, 1976a), it is surprising that it is relatively unaffected by Con A in its adsorption to strain U440, compared with phage Br2. If the initial adsorption of phage T4 is via the long tail fibres (Simon & Anderson, 1967; Wilson, Luftig & Wood, 1970), these may be better able to interact with glucose residues within the LPS, in the presence of Con A, than the capsid spikes of Br2 which is an icosahedral phage. Another possible explanation

Fig. 4. The effect of Con A on the uptake of [^3H]actinomycin D by EDTA-treated and untreated cells of *E. coli* K12 mutant strain U440: ●, untreated; ○, treated with Con A; ▲, treated with EDTA; △, treated with EDTA and Con A.

may be that phage T4 and Con A interact with different carbon atoms of the same glucose molecule.

It has recently been reported that Con A blocks adsorption of phage T4 to *E. coli* B (Watanabe, 1976), a strain in which a glucose residue is terminal in the LPS (Prehm *et al.*, 1976a). A higher concentration of Con A (6 mg ml^{-1}) was used than in the present study.

Cell agglutination using a fatty-acid auxotroph

Agglutination of animal cells by Con A is associated with a change in the topographic distribution (clustering) of Con A binding sites (Nicolson, 1972a, b). Such clustering of the binding sites necessarily entails movement in a fluid membrane, and the data indicate that clustering is necessary for agglutination to occur. In an attempt to determine if the same situation pertains in the outer membrane of *E. coli*, strain K1060-2/UCB19 (a fatty-acid auxotroph which is agglutinated by Con A) was grown with a *cis* $\text{C}_{18:1}$ fatty acid (oleic acid) or a *trans* $\text{C}_{18:1}$ fatty acid (elaidic acid) as its fatty acid supplement. The transition temperatures of phospholipids containing these fatty acids are approximately 15 and 37 °C respectively (Overath *et al.*, 1971). Bacteria grown on oleate or elaidate were thus incubated at 10 and 39 °C and Con A was added as before. Both the oleate- and elaidate-grown bacteria agglutinated with Con A at both the high and low temperatures. These results suggest that there is no requirement for clustering of Con A binding sites in order to produce agglutination, but do not indicate whether or not clustering occurs.

Horwitz, Hatten & Burger (1974) have done similar experiments with animal cells. In contrast to our results they found that growth with elaidate increased, and oleate decreased, the thermal transition temperature for Con A agglutination; below the transition temperature for agglutination (26 to 28 °C for elaidate-grown bacteria), little agglutination was observed.

Possible membrane damage by Con A

LPS profoundly influences, directly or indirectly, the permeability of the outer membrane since loss by mutation or by EDTA treatment increases permeability (Leive, 1974; Boman, Nordström & Normark, 1974; Nikaïdo, 1976). Thus if LPS molecules are mobile within the outer membrane, as newly synthesized molecules are (Mühlradt *et al.*, 1974; Bayer, 1975; Kulpa & Leive, 1976), Con A might cause 'clustering' of LPS resulting in areas of membrane depleted of LPS, which might, in turn, result in greater permeability.

We considered the effect of Con A on leakage of a large molecule, alkaline phosphatase, through the outer membrane from the periplasm, and the effect on permeability to relatively small molecules such as Gentian violet and actinomycin D. No appreciable leakage

of alkaline phosphatase, in excess of that of the controls, was caused by Con A attachment (data not shown). Similarly, with Gentian violet, we observed type I uptake (Gustafsson *et al.*, 1973) in both controls and Con A-treated cells, but Con A did not appear to cause any type II uptake (data not shown). In studies on the entry of actinomycin D into the cell (Fig. 4), treatment with EDTA caused uptake, as demonstrated by Voll & Leive (1970), but Con A had no effect on either EDTA-treated or untreated cells. No attempt has yet been made to establish directly whether clustering of LPS occurs, but these results can be interpreted to indicate that it does not.

Concanavalin A may be useful in the purification of LPS from the mutant strains of *E. coli* K12 reported here, by affinity chromatography using Con A-Sepharose, a technique successfully used for the purification of teichoic acids (Doyle, Birdsell & Young, 1973). This possibility is of particular interest in view of the characterization of protein covalently-linked to the LPS of *E. coli* by Wu & Heath (1973).

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