and the orientation of one of the morphological units is changed by about 20° between patterns. It is possible that the two patterns may reflect a structural transformation of the layer, and may be related to a change in permeability of the A-layer. The structural gene for the subunit protein has been cloned using the vector λgt11. The protein expressed by this gene has the same subunit MW as the native mature exported A-proteins. Initial molecular genetic evidence indicates that the A-protein gene is highly conserved in the species *A. salmonicida*.

Transposon mutagenesis has provided a range of single-insertion mutants with altered ability to produce the A-layer. One Tn5 mutant accumulated A-protein in the periplasm, and did not produce an A-layer[1]. Virulence assays showed that this mutant had lost its ability to kill fish confirming the requirement of an assembled A-layer for virulence. The layer contributes to serum resistance[6], and appears to have an important role in the interaction of *A. salmonicida* with macrophages[8]. Interestingly, the Tn5 mutant which accumulated A-protein in the periplasm appeared to be unaltered in its ability to produce and export proteins such as hemolysin and protease, suggesting that the export pathway for A-protein may be unique.

Strains of *Aeromonas hydrophila* which exhibit high virulence for fish also produce a tetragonally arrayed surface layer composed of a protein of subunit MW 52,000[4]. Similar layers also occur on a number of strains of *A. hydrophila* isolated from clinical disease in humans and other mammals. The array protein from one strain of *A. hydrophila* has been purified and characterized. The *A. hydrophila* protein is similar in overall amino acid composition to the *A. salmonicida* A-protein, but differs with respect to the N-terminal amino acid sequence. Immunochemical analysis shows that the *A. salmonicida* and *A. hydrophila* proteins differ antigenically. The *A. salmonicida* protein appears to be antigenically conserved while the *A. hydrophila* array proteins display antigenic diversity.

Surface array-producing strains of both species of *Aeromonas* also produce a lipopolysaccharide (LPS) which is characterized by the presence of O-polysaccharides of unusually homogeneous chain length[2,3]. This morphological form of LPS appears to be important for the assembly of the surface protein array. This is well illustrated by a Tn5-insertion mutant which is unable to produce a smooth-type LPS with its characteristic O-polysaccharides of homogeneous chain length, but still retains the ability to produce and export A-protein. When grown in liquid media these cells are unable to assemble A-layer on their surface resulting in the excretion of large amounts of A-protein into the culture media. The chain length of the O-polysaccharide of *A. salmonicida* contains 16 to 18 repeat units while that of *A. hydrophila* contains approximately 10 repeat units. The O-polysaccharide chains protrude through the surface arrays and contribute to the serum resistance of both species. The O-polysaccharides are also major surface antigens. The O-chains of *A. salmonicida* are antigenically conserved, as are the homologous length O-chains of the *A. hydrophila* LPS. Although the chemical composition of the *A. salmonicida* and *A. hydrophila* homologous length O-polysaccharides are different, both species of O-chains carry an antigenically cross-reactive epitope, as well as a species serospecific epitope. Other strains of *A. hydrophila* produce an LPS with O-polysaccharides of heterologous length. These O-polysaccharides are not antigenically cross-reactive with the homologous chain length O-polysaccharides. Such strains of *A. hydrophila* appear not to produce S-layers.

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Aeromonas adhesin antigens

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Key words. Adhesion-adhesins; *Aeromonas* antigens; haemagglutinins.

Many strains of *Aeromonas* species (aeromonads) produce adhesins[4] which are readily detected using simple haemagglutination assays, and although there is yet little convincing evidence that adhesins are virulence factors for aeromonads, it seems probable that they do play a role in aeromonad disease. For example, diarrhoeal isolates of motile aeromonads co-produce Vero cell cytotoxin and HAG significantly more frequently than do non-diarrhoeal faecal isolates (table).

Both the motile and non-motile aeromonads produce haemagglutinins (HAG)[4,10]. There is a large diversity of HAG types, as demonstrated by what species of erythrocytes are agglutinated, co-aggregation with yeast, and carbohydrate inhibition assays. On this basis alone approximately 40 different types have been recognised[2]. The numbers of HAG types may be extended further by using co-aggregation studies with *Salmonella* strains which have known lipopolysaccharide (LPS) structure[3].

Of the *Aeromonas* HAG's studied to date, all apear to be protein[1,4]. At first it was thought that the HAGs might be associated with fimbriae because some of the early work on *Aeromonas* HAGs revealed the presence of fimbriae on haemagglutinating strains[4]. Subsequent work with the fimbriated strain (A6)

Relationship between diarrhoea-association and co-production of cytotoxin and haemagglutinin by 135 *Aeromonas* strains isolated from human faeces. $p > 0.005$ (Fisher's exact test)

	Diarrhoea-associated	Not diarrhoea-associated	
Co-production positive	87	4	91
Co-production negative	26	18	44
	113	22	135

showed that, in this case, the production of fimbriae and HAG are independent characteristics. For example, A6 can be grown under conditions where it produces HAG but does not produce fimbriae; conversely, when grown under a different set of conditions, it will not haemagglutinate, but is fimbriate. Furthermore, there are several strains of *Aeromonas* on which fimbriae have never been demonstrated, but which haemagglutinate well. Some of these non-fimbriate strains produce a HAG which seems to be the same, serologically, as that produced by A6. The evidence for this comes from studies with a polyclonal antiserum (3/83) which was made by immunising a rabbit with live cells of A6. This serum not only inhibits the HAG of A6, but also the HAGs of many nonfimbriated strains. However, whilst the fimbriae of A6 do not appear to have a role to play in haemagglutination, it is possible that they are involved with attachment to other cell types which express surface antigens differing from those of erythrocytes.

As at least some *Aeromonas* HAGs appeared to be non-fimbrial, investigations were carried out to establish with what cell components they might be associated. In initial studies we were unable to produce any cell fraction which had convincing HAG properties. Purified fimbriae did not haemagglutinate, nor did they attach to erythrocytes or erythrocyte membranes. However, we were able to show that outermembrane vesicles prepared from one strain (A16) would attach to erythrocyte membranes; this attachment could be inhibited, indeed reversed, by L-fucose, the only sugar which will inhibit the HAG of A16. In view of this finding, it seemed most likely that the *Aeromonas* HAG would be found in the outer membrane fraction of the bacterial cell. Strain A6 was chosen to begin the search for the HAG. There were two reasons for this choice. Firstly, the HAG-neutralising serum 3/83 was available, and secondly, the A6-type HAG is relatively common in aeromonads isolated from clinical and environmental sources.

SDS-PAGE[8] and Western blot[9] techniques were used to identify and locate the HAG protein of A6. As the HAG of A6 is well-expressed at 37 °C, and 43 °C-grown cells do not haemagglutinate, solubilised whole-cell extracts and outer-membrane (OM) preparations of 37 °C-grown and 43 °C-grown bacteria were analysed with serum 3/83. Proteinase-K-digested preparations of solubilised whole-cell and OM were similarly analysed. From this series of experiments little difference between 37 °C-grown and 43 °C-grown bacteria was found, except that there is a component of apparent molecular mass 43 kDa present in 37 °C-grown but not 43 °C-grown cells. This is protein as it is not in proteinase-K-digested preparations. The 43 kDa protein is prominent in both whole-cell and OM preparations (fig. 1). In addition, other strains of *Aeromonas* whose HAG could be inhibited by 3/83, also had a 43 kDa protein which reacted in Western blots with 3/83 (data not shown).

Further evidence that the HAG activity of A6 was associated with an outer membrane protein (OMP) of apparent molecular mass of 43 kDa was obtained with absorbed serum 3/83. In view of the fact that 43 °C-grown cells lacked the 43 kDa OMP, it was reasoned that 43 °C-grown cells might absorb out antibodies other than those directed against the HAG. Accordingly, serum 3/83 was absorbed with 43 °C-grown cells of A6. The resultant serum (4/83) was able to inhibit the HAG of A6 and the HAG of other strains possessing the A6-type HAG. When used in Western blots, serum 4/83 reacted strongly with two components of A6 only. These were proteins of molecular mass 43 kDa and 37kDa respectively. They were both present in the whole-cell lysate, but only the 43Kd component was detectable in the OM preparation. No activity was detected in the proteinase-K-digested materials, indicating that no antibody to LPS remained in serum 4/83 (fig. 1).

Currently, attempts are being made to produce mouse monoclonal antibody which has neutralising activity against the A6 HAG. Should this be successful these antibodies will be used in an attempt to confirm the identity of the 43 kDa protein. These monoclonal antibodies would then be used in affinity chromatography experiments to isolate and purify the 43 kDa protein from A6 cell fraction.

The HAG of A6 agglutinates human, but not horse erythrocytes, and the HAG can be inhibited by L-fucose[4]. Furthermore, *Salmonella* co-aggregation studies have shown that terminal L-fucose is a vital part of the A6 HAG receptor molecule. Also, it has been shown (Savvas and Atkinson – unpublished observations) that A6 does not agglutinate erythrocytes of the rare 'Bombay' phenotype of the human ABH(O) blood group system; all other ABH(O) phenotypes are agglutinated by A6. 'Bombay' erythrocytes lack the H antigen which is present on all other ABH(O) phenotypes (fig. 2). 'Bombay' erythrocytes differ from other ABH(O) in that they lack the terminal L-fucose residue of the H antigen. From the above observations we propose that the erythrocyte receptor for the A6 HAG is the H antigen.

If one accepts the proposition that the A6 HAG does enable the bacterium possessing it to attach to cells which express H antigen on their surfaces, what advantage or impediment might this impart to such a cell? It is well-established that *Aeromonas* strains may produce enterotoxins[6], and it is probable that, like the cholera bacillus, these toxins need to be delivered directly to the target-cell surface for maximal effect to occur. As H antigens are expressed on the surface of intestinal cells[7] it seems likely that strains possessing the A6 HAG will attach to intestinal cells. On the other hand, if H antigens are present on intestinal mucus, the mucus might entrap the HAG-producing bacterium, thus denying the bacterium access to the surface of the intestinal epithelium.

Other *Aeromonas* strains produce HAGs which have different

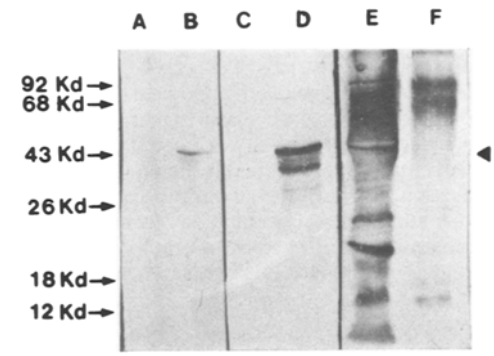

Figure 1. Western blot analysis of preparations of *Aeromonas* strain A6. A6 preparations were electrophoresed on 12.5% SDS-PA gels, then electrophoresed onto nitrocellulose paper. Serum 3/83 was produced by immunising a rabbit with live cells of strain A6 grown at 37 °C. Serum 4/83 was produced by absorbing 3/83 with A6 grown at 43 °C. Both 3/83 and 4/83 inhibit the haemagglutinin of A6. Lane A: outer membranes of 43 °C-grown A6; Lane B: outer membranes of 37 °C-grown A6; Lanes C, F: whole cells of 37 °C-grown A6, proteinase-K-digested; Lanes D, E: whole cells of 37 °C-grown A6. Lanes A, B, C and D were treated with serum 4/83. Lanes E and F were treated with serum 3/83. ◀Haemagglutinin protein: a 43 kDa protein of the bacterial outer membrane.

'Bombay' cell	β-Gal-(1→3)-GNAc-	or	β-Gal-(1→4)-GNAc-
H antigen	β-Gal-(1→3)-GNAc-	or	β-Gal-(1→4)-GNAc-
	2		2
	|		|
	|		|
	1		1
	α-Fuc		α-Fuc

Figure 2. The structure of the terminal sugars of ABH(O) blood group antigens[5]. Gal, D-galactose; GNAc, N-acetyl-D-glucosamine; Fuc, L-fucose.

characteristics from those of A6. For example, strain A7 will agglutinate with both human and horse erythrocytes, the haemagglutination is inhibited by D-galactose, and it co-aggregates with *Salmonella* strains which have terminal D-galactose residues on their LPS. In contrast to A6, A7 is able to agglutinate with 'Bombay' erythrocytes. Neutralising antibodies have been produced against the A7 HAG, and studies similar to those described above for A6 are under way.

Finally, there is an interesting distribution of adhesin type among the motile aeromonads. The A6-type adhesin was found in 14 of 59 *A. hydrophila* (24%), 1 of 35 *A. caviae* (3%), and 2 of 61 *A. sobria* (3%). Another HAG type, characterised by being inhibited by mannose but not fucose or galactose, was found in 4 of 59 *A. hydrophila* (7%), none of 35 *A. caviae*, and 25 of 61 *A. sobria* (41%). These results suggest that there may be significant differences in the distribution of HAG types between the different species of aeromonads. This possibility is currently under investigation.

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Concluding remarks: Areas of future research

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Key words. Aeromonas; Plesiomonas.

This workshop reviewed a variety of aspects of ongoing *Aeromonas* and *Plesiomonas* research and pinpointed several areas in which further research seems particularly urgent. These include:

1. Comparative studies on sensitivity and specificity of various selective media. It was proposed that this should involve a multicentric investigation. Carrier rates in various countries among both indigenous and transient populations would also be the product of such a collaborative study. At the time of printing, part of these studies may already be in progress (inquire with the senior author).
2. Follow-up studies on carriers.
3. Determination of the precise role of *Aeromonas* and *Plesiomonas* in diarrhea. While epidemiological data point to these bacteria being etiological agents of diarrhea, it was the consensus of opinion at the workshop that the evidence was still not conclusive. Studies could investigate correlations between diarrhea and *Aeromonas/Plesiomonas* serotypes, biotypes, serum sensitivity, optimal growth temperatures, number of organisms per gram of stool, presence of known toxins (synergistic effects?), hemagglutinins, adhesins, and various underlying conditions in an endeavor to establish the enteropathogenicity (or lack of it) of these species. Further volunteer feeding experiments may be called for. Recognizable markers of enteropathogenicity would be looked for in these studies.
4. The effect of antimicrobial treatment upon *Aeromonas/Plesiomonas*-associated diarrhea.
5. The role of *Aeromonas* and *Plesiomonas* in *chronic* diarrhea and colitis.
6. Characterization of reference strains.
7. Standardization of media and of procedures for testing biochemical reactions and possible virulence factors.

It was also agreed that a centralized literature collection would be desirable. At the time of publication of this symposium, plans will have been worked out (for inquiries please contact the senior author).

A second International Workshop on *Aeromonas* and *Plesiomonas* is planned for 1988.

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