PATHOGENICITY

Temperature-dependent protein and lipopolysaccharide expression in clinical *Aeromonas* isolates

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Summary. Clinical isolates of *Aeromonas* were grown at eight different temperatures from 10°C to 40°C. Whole cell lysates were examined by SDS-PAGE and major temperature-dependent changes to both protein and lipopolysaccharide (LPS) profiles were identified. Cells grown at the higher temperatures (37°C and 40°C) produced abundantly a protein of c. 60 kDa which was not detected at the lower temperatures. Temperature-dependent expressions of other proteins were also noted but these were more variable among the isolates. An effect of temperature on expression of lipopolysaccharides was also noted in that some strains produced significantly less O-polysaccharides at the higher temperatures. After fractionation of cells, major differences in the expression of cell envelope and outer-membrane proteins between cells grown at low and high temperatures were noted although no unifying patterns could be discerned. Such growth temperature-induced changes in the cell envelope constituents have not been described previously for *Aeromonas* isolates from man.

Introduction

Motile members of the genus *Aeromonas*, which are gram-negative bacilli within the family Vibrionaceae, are common in many different environments, particularly fresh waters and estuaries. They have also been isolated from a wide variety of fresh and packaged foods. Interest in these organisms has been increasing steadily since they have been recognised as human enteric pathogens.^{1,2} Three phenospecies of motile aeromonads are currently recognised—*A. hydrophila*, *A. caviae* and *A. sobria*—and all have been associated with diarrhoeal disease. The taxonomy of the group is complex and requires genetic analysis to define 13 genospecies. However, given the technological difficulties in performing such studies, clinical isolates are still only identified to the phenospecies level. In many instances the presence of motile aeromonads is all that is reported and further phenospecies identification is not pursued.

Isolates from cases of human gastrointestinal disease have been shown to produce various biologically active extracellular products such as haemolysins,³ cytolytic and cytotoxic enterotoxins,⁴ and degradative enzymes (acetylcholinesterase, phospholipases and proteases).^{5,6} The function of these products has been

documented *in vitro* but their contribution *in vivo* to the disease process is yet to be defined. Also, cell surface components have been recognised to contribute to the colonisation of animal tissues by many bacterial pathogens. Lipopolysaccharide (LPS) O-antigens, regular surface protein layers, fimbriae and various outer-membrane (OM) proteins all appear to be associated with the pathogenic potential of the motile aeromonads.⁷ Thus, the exact virulence mechanisms employed by *Aeromonas* spp. involved with gastrointestinal illness are by no means clear, but it is likely that both cell surface and extracellular factors play important roles.

Many bacterial virulence mechanisms are under environmental regulation by temperature, nutrient limitation, atmospheric composition or pH. This enables the bacterial pathogen to co-ordinate expression of virulence factors with the availability of a suitable target tissue. Regulation of virulence thus represents a considerable metabolic saving for a bacterial pathogen. Recognition of environmental signals responsible for triggering the expression of virulence factors has allowed the identification of certain virulence mechanisms and facilitated their detailed characterisation. The virulent phenotype of *Shigella* spp. is temperature-regulated such that strains grown at 37°C are fully invasive and penetrate mammalian cells, whereas the same strains grown at 30°C fail to invade the target cells and are, therefore, non-

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virulent.⁸ This ability has been associated with the differential expression of OM proteins. Analogous systems have been recognised in strains of *Yersinia enterocolitica* and *Y. pseudotuberculosis*.⁹

Some factors must influence the ability of aquatic aeromonads, living at relatively low temperatures, to adapt successfully to the relatively high temperatures of a human host. A number of studies have attempted to address the effects of temperature on the production of extracellular enzymes and on selected metabolic properties.¹⁰⁻¹² As yet no clear patterns of temperature-dependent regulation of these virulence factors have been defined. In contrast to the interest in temperature regulation of extracellular components, there have been only a few reports on how temperature affects cell surface and OM components. This is all the more remarkable given the growing interest in the contribution of this region of the cell to virulence. The only report on the effect of temperature on the OM of *Aeromonas* spp. concerns a single serogroup of *A. hydrophila* in which a small number of strains from various sources was examined.¹³ The purpose of this study was to examine how growth temperature influences protein and LPS expression among *Aeromonas* isolates from cases of human diarrhoeal disease.

Materials and methods

Bacterial strains

The *Aeromonas* strains studied were supplied by Professor C. A. Hart, University of Liverpool. They were isolates from children with gastro-enteritis from whom no other pathogen was identified. Stock cultures were maintained at -70°C in Trypticase Soy Broth (TSB; BBL Microbiology Systems, Cockeysville, MD, USA) containing glycerol 15% v/v. All the strains were grown at chosen temperatures in the range $10-40^{\circ}\text{C}$ for 24-48 h on Tryptone Soya Agar (TSA).

SDS-PAGE

SDS-PAGE of proteins and LPS was performed by the discontinuous buffer system of Laemmli.¹⁴ A vertical slab cell system (BioRad Laboratories, Richmond, CA, USA) was used for all separations. Samples were boiled for 10 min in SDS-PAGE solubilisation buffer (glycerol 10%, β -mercaptoethanol 5%, SDS 3%, bromophenol blue 0.01% in 62.6 mM Tris, pH 6.8). Gels were run at 40V through acrylamide 4.5% stacking gels and at 60V through acrylamide 12.5% or 10% separating gels. With this system run times of 20-24 h were achieved.

Analysis of LPS

The electrophoretic migration pattern of LPS was determined by a modification of the procedure of Hitchcock and Brown.¹⁵ Cells were boiled for 10 min

in the SDS-PAGE solubilisation buffer at a ratio of 1 mg (wet weight) of cells to 100 μl of buffer. The lysates were then digested with proteinase K at 60°C for 1 h at a ratio of 1000 μl of original cell lysate to 100 μl of a 1 mg/ml solution of proteinase K. Samples (30 μl) were loaded on to SDS-PAGE gels and LPS was detected by silver staining.¹⁶

Analysis of whole cell proteins

Each strain was passaged twice at chosen temperatures. The cells were harvested and boiled in the SDS-PAGE solubilisation buffer (1 mg wet weight/100 μl) for 10 min. Samples (30 μl) were applied to SDS-PAGE gels and protein bands were detected by staining with Coomassie Brilliant Blue R250.

Fractionation of cells

Cells were harvested by centrifugation at 4400 *g* for 30 min at 4°C and washed twice in 20 mM Tris-HCl, pH 7.4 (Tris buffer). The washed cells were re-suspended in Tris buffer (10 g wet weight cells/50 ml buffer) and disrupted by shaking with glass beads (Braun Cell Disruptor) for 2 min. The unbroken cells were deposited by centrifugation at 4400 *g* for 30 min at 4°C , leaving a supernate containing cell envelopes.

Isolation of outer membranes (OM) and inner membranes (IM)

The total cell envelope fraction was collected by centrifugation at 31400 *g* for 30 min at 4°C . OM were prepared from isolated cell envelopes by differential solubilisation of the IM with sodium lauryl sarcosinate by the method described by Filip *et al.*¹⁷ The total membrane protein: detergent ratio used was 1:6 (mg:mg). The samples were shaken for 30 min at room temperature and then centrifuged at 31400 *g* for 30 min at 4°C . The OM pellet was washed twice in Tris buffer and the supernate containing IM was concentrated by freeze-drying. The protein concentration was estimated by the Bradford assay method.¹⁸

Results

Electrophoretic analysis of whole cell proteins

To determine the effect of the growth temperature on protein expression, whole cell lysates of 29 clinical isolates of *Aeromonas* grown at eight different temperatures ($10-40^{\circ}\text{C}$) were analysed by SDS-PAGE. As has been reported previously, different patterns of whole cell protein expression were noted when profiles of strains grown at the same temperature were compared.^{19,20} In contrast, nearly all the strains expressed a common temperature-dependent protein of c. 60 kDa that was produced in greater amounts at higher temperatures (37°C and 40°C) than at lower temperatures. The whole cell protein profiles of two representative strains grown at the different tempera-

Fig. 1. SDS-PAGE (acrylamide 10%) of whole cell proteins from *Aeromonas* strains UU45 (a) and UU38 (b) grown at different temperatures. The growth temperatures were: lane 1, 10°C; 2, 15°C; 3, 20°C; 4, 25°C; 5, 30°C; 6, 35°C; 7, 37°C; 8, 40°C. Standard mol. wt marker proteins are included to the right of the gel: 97.4, 66, 45, 31 and 21.5 kDa. The strains had a 60-kDa protein that was more intense at high temperatures (37°C and 40°C) than at lower temperatures (upper arrow). A minor temperature-regulated protein of 26 kDa was also seen in strain UU38 (b: lower arrow).

tures are shown in fig. 1. Based on the intensity of Coomassie Brilliant Blue staining, the 60-kDa protein was the most abundant protein produced at the higher temperatures. This was not the only temperature-dependent protein visible in whole cell lysates. Some strains also expressed a 26-kDa protein only at high temperature. In contrast, some isolates strongly expressed a 40-kDa protein at low temperatures (results not shown).

Some smearing was detected among the protein profiles of 12 isolates. This prevented the resolution of proteins of *c.* 45–60 kDa. Previous investigations^{21,22} had indicated that smearing of protein bands was due to the presence of a particular type of LPS organisation that was often linked to virulence in certain strains. In this study, the existence of the smear regions was found to be related to the effect of the growth temperatures because the intensity of the smear

Fig. 2. SDS-PAGE (acrylamide 10%) of whole cell proteins from *Aeromonas* strains UU40 grown at different temperatures. Whole cell lysates were stained with Coomassie Brilliant Blue after electrophoresis. The growth temperatures were: lane 1, 10°C; 2, 15°C; 3, 20°C; 4, 25°C; 5, 30°C; 6, 35°C; 7, 37°C; 8, 40°C. Standard mol. wt marker proteins are included to the right of the gel: 97.4, 66, 45, 31 and 21.5 kDa. A smear, obscuring resolution in the 45–60-kDa range, can be observed in all lanes except lanes 7 and 8, where cells grown at the higher temperatures are analysed.

decreased, and sometimes even disappeared, at higher temperatures. A representative strain whose profile exhibited smearing is shown in fig. 2. The smeared regions are prominent at the lower temperatures (10–30°C) but slightly or not at all at higher temperatures. The resolution of the proteins of *c.* 60 kDa was obscured at lower temperatures but at the higher temperatures, when the smear was greatly reduced, a major band of *c.* 60 kDa could be clearly identified.

Electrophoretic analysis of LPS

Temperature-dependent smearing of protein profiles suggested a modification of the amount or type, or both, of LPS formed at different growth temperatures. The organisation of LPS in *Aeromonas* clinical isolates was analysed by treatment with proteinase K and silver staining. Three patterns of O-polysaccharide side chains were found in the 74 strains investigated: homogeneous chain length (18%), heterogeneous chain length (54%) and absence of O-polysaccharides (28%) (fig. 3). Fifteen randomly selected isolates were analysed to determine the effect of growth temperature on their LPS expression. A temperature effect on the organisation of LPS in the *Aeromonas* cell envelope was not seen; identical patterns of LPS organisation were present at all growth temperatures. However,

Fig. 3. SDS-PAGE of LPS from *Aeromonas* strains grown at 37°C. Proteinase K-digested whole cell lysates were silver stained after electrophoresis. Three patterns of O-polysaccharide side-chains were present among the LPS molecules: A, homogeneous chain length—1 (UU60), 2 (UU62) and 6 (UU66); B, heterogeneous chain length—lanes 3 (UU63), 4 (UU64) and 7 (UU67); C, lack of O-polysaccharide—lane 5 (UU65).

for some strains the concentration of the O-polysaccharide-substituted fraction of the LPS, based on the intensity of staining of the bands on the gels, was lower at higher temperatures (fig. 4). Furthermore, the existence of the smear region on the protein profiles was found to be related to the presence of the O-polysaccharide side chains. In the case of strain UU36 (fig. 4), O-polysaccharide side chains were poorly expressed at 37°C and 40°C and, consequently, no smearing of the protein profiles was noted at those temperatures. Smearing was apparent when the cells were grown at lower temperatures and electrophoretic analysis of LPS showed the presence of an extensive homogeneous chain length O-polysaccharide substituted fraction.

Subcellular localisation of temperature regulated proteins

Growth temperature-dependent alterations in protein and LPS production suggested that some strains may alter their cell surfaces in response to temperature. This prompted an examination of cell surface components after fractionation of cells grown at different temperatures. To determine the location of the temperature-regulated proteins, different subcellular fractions were prepared including total cell envelope, OM and a cytoplasmic-periplasmic fraction. These were analysed by SDS-PAGE. Three representative

isolates (UU28, UU36 and UU38) were chosen for this analysis. An analysis of strain UU36 is shown in fig. 5. A protein of *c.* 60 kDa was found in both the cytoplasmic-periplasmic and envelope fractions. This protein could not be found in the OM and, as expected, it was detected only at 37°C. Significantly, there were major temperature-related differences in the expression of some envelope proteins that were not apparent in whole cell profiles. The enrichment of envelope proteins afforded by the fractionation of the cells allowed these to be detected. One major 35-kDa protein was present in the cell envelope fraction at 15°C but not at 37°C (fig. 5, lanes 3 and 4). A protein of similar mol. wt was detected in the OM fraction at 37°C but not at 15°C. This protein was also present in the envelopes of strains UU28 and UU38 (results not shown). Once again the protein was formed only at 15°C, although in the case of these two strains a protein of similar mol. wt was found in the OM fraction as well as in the envelope fraction. Based on Coomassie Brilliant Blue staining intensity, this was the major cell envelope protein expressed at lower temperatures.

Some variation in the expression of other proteins by strain UU36 was also detected. Envelope proteins of *c.* 42 and 52 kDa were expressed preferentially at lower temperatures. A 42-kDa protein with similar temperature-dependent expression was also seen in the OM fraction, although no corresponding 52-kDa protein could be identified. OM proteins of 25 and 48 kDa were expressed preferentially at 37°C. Analogous effects were also observed for strains UU28 and UU38 (results not shown). Thus, there is a major difference in the quantitative and qualitative expression of cell envelope and OM components between cells grown at low (15°C) and high (37°C) temperatures.

Discussion

The factors influencing the ability of aeromonads living at relatively low temperatures in an aquatic habitat to adapt successfully to environments of relatively high temperatures, i.e., vertebrate hosts, are not fully understood. The question then arises as to whether or not it is possible for these organisms to express some virulence factors only when they are in contact with a warm-blooded host. This could enable an organism living in a nutrient-poor aquatic environment to save energy. Thus the identification of such temperature-regulated components may help to promote an understanding of virulence mechanisms among the motile aeromonads. Schubert and Matzinou²³ showed that when low temperature-adapted *Aeromonas* spp. were exposed to high temperatures, they grew faster than those already adapted to higher temperatures. This ability would probably be advantageous for aquatic strains that suddenly encounter a homeothermic host. Many researchers are interested in the effects that temperature may have on bacterial cellular chemistry, particularly as it affects

a

1 2 3 4 5 6 7 8

b

1 2 3 4 5 6 7 8

Fig. 4. SDS-PAGE (acrylamide 10%) of whole cell proteins and LPS from *Aeromonas* strain UU36 grown at different temperatures. After electrophoresis whole cell lysates were stained with Coomassie Brilliant Blue to identify proteins (**a**) and proteinase K-digested whole cell lysates were silver stained to detect LPS (**b**). The growth temperatures were: lane 1, 10°C; 2, 15°C; 3, 20°C; 4, 25°C; 5, 30°C; 6, 35°C; 7, 37°C; 8, 40°C. Standard mol. wt marker proteins are included to the right of gel (a): 97.4, 66, 45, 31 and 21.5 kDa.

membrane lipids, LPS and OM proteins, as well as their relationship with virulence. Members of the family Enterobacteriaceae and the genus *Pseudomonas* have been studied for the effect of growth temperature on their LPS chemistry. Changes in expression of the O-polysaccharide region of the LPS have been noted at different temperatures. However, there is significantly less information available for members of other bacterial families, including the Vibrionaceae. Although there is no unequivocal evidence, it seems that water and fresh foods may be the major source of

aeromonads for human infections. Thus, the ability to adapt to environments of different temperature may be of great significance to these organisms.

This study has provided some of the background information necessary to investigate the effect of temperature on the virulence of motile *Aeromonas* strains from human diarrhoeal disease. The investigation revealed that clinical isolates had variable whole cell protein patterns and no groupings could be defined. The use of whole cell protein profiles in the typing of *Aeromonas* spp. has recently been shown not

Fig. 5. SDS-PAGE (acrylamide 12.5%) of whole cell proteins and subcellular fractions of *Aeromonas* strain UU36 grown at different temperatures. Cells were grown at 15°C (lanes: 2, 4, 6 and 8) and 37°C (lanes: 1, 3, 5 and 7). The fractions are loaded as follows: whole cell lysates, lanes 1, 2; cell envelope, 3, 4; OM, 5, 6; cytoplasmic-periplasmic fraction, 7, 8. A 60-kDa protein is present in whole cell lysates, envelopes and the cytoplasmic-periplasmic fraction of cells grown at 37°C but not at 15°C. This band is absent from the OM fraction (arrow). A 35-kDa protein is the most abundant protein species in the envelope fraction at 15°C but is not prominent at 37°C (arrow). Standard mol. wt marker proteins are included to the left of the gel: 97.4, 66, 45, 31, 21.5 and 14.5 kDa.

to correlate with the phenotype of the isolates.²⁴ This variability undoubtedly reflects the heterogeneous nature of the genus *Aeromonas*. Groupings of strains on the basis of whole cell protein patterns may become possible when fingerprints of members of the same genospecies can be compared. However, this is not likely until techniques for identification of genospecies are widely available and performed routinely.

A high percentage (28%) of isolates appeared to lack O-polysaccharides. There have been no previous reports of such a common LPS organisation among human clinical isolates. In fact, most investigators have regarded the presence of an O-polysaccharide as a necessary virulence factor for this group of organisms.⁷ The functional significance of this observation remains to be determined and will have to await the comparison of different strains in standard virulence assays. Non-invasive, enterotoxigenic strains of *Aeromonas* are unlikely to require an extensively O-polysaccharide substituted LPS to initiate a transient colonisation of gastrointestinal cells. Most of the evidence implicating O-polysaccharide as a virulence factor comes from measurements of complement killing rates *in vitro*. The ability to avoid complement is likely to be important only for invasive strains and those that only colonise the mucosal surface are unlikely to benefit from the presence of such a

structure. Correlation of adhesive abilities, invasive abilities and resistance to complement-mediated cell lysis among the isolates expressing different O-polysaccharide organisations is under way.

The presence of a group of strains with homogeneous chain length O-polysaccharides was also important as it suggested that a significant percentage of human isolates may possess an S-layer. Both Dooley *et al.*^{21,22} and Kokka *et al.*²⁵ have documented the relationship of this LPS organisation to possession of an S-layer. The function of most S-layers identified to date remains an enigma, although participation in resistance to complement-mediated cell lysis, avoidance of intracellular killing within macrophages, and adhesion to cell surfaces, have all been demonstrated for analogous proteins in other systems. The presence of an S-layer in a strain of *A. hydrophila* isolated from a case of septicaemia was thought to contribute to the invasiveness of the organism, although a mechanism was not identified.²⁶ Examination for S-layers on this group of human diarrhoeal pathogens is to be made by electronmicroscopy and by biochemical extraction.

The results of the analyses carried out at different temperatures revealed that there were major differences in expression of LPS and protein within isolates. Recently, Merino *et al.*¹³ reported that the effect of temperature on strains of *A. hydrophila* serotype O34 was on the growth rate, the generation time being longer at low temperature (20°C) than at high temperature (37°C). The present study also indicated that the growth rate appeared longer at low temperature and the growth temperature also affected the morphology of the colonies on solid media. The optimal growth temperature for *A. hydrophila* is considered to be 28°C.²⁷ However, Statner *et al.*¹⁹ have shown that the optimal growth temperature of motile *Aeromonas* species is not necessarily 28°C and that better growth, manifested by a shorter lag phase, a higher rate of log-phase growth, or both, can occur at 37°C. Merino *et al.*¹³ also reported on the effects of temperature on OM proteins and LPS in a small number of fish and animal isolates. These investigators found that O-polysaccharide production was switched off at 37°C but not at 20°C. The results from the large number of strains examined here indicate that, in many cases, LPS O-polysaccharide expression is reduced at higher temperatures but it does not cease. Even when strains were grown at 40°C, O-polysaccharides could be identified on the silver stained gels.

In contrast to the differences in LPS expression, Merino *et al.*¹³ found no major differences in OM protein expression between cells grown at different temperatures. This is contrary to our findings. In many cases the major proteins present in the OM were completely different when cells from two different growth temperature ranges were compared. For example, the 25- and 48-kDa proteins were two of the most abundant OM proteins at 37°C but were much less prominent at 15°C. The production of these species

must have a major influence on the surface chemistry of the cells. Furthermore, the expression of other major components of the cell envelope was temperature-dependent and, while they did not co-purify with the OM fraction, it is possible that they could make a significant contribution to cell-surface properties. Virulence assays that measure cell surface properties will be used to assess some of these functional differences in cell surface properties.

The discrepancy between two studies of the OM properties of *Aeromonas* spp. grown at different temperatures is probably not too surprising given the taxonomic difficulties in identifying strains. In addition, the studies of Merino *et al.*¹³ involved fewer strains and a narrower temperature range. This study involved a broader temperature range (from 10°C to 40°C) and a larger number of strains (29) from a

similar environment. The strains examined by Merino *et al.*¹³ showed greater virulence for mice and fish when grown at 20°C than when grown at 37°C. This would be appropriate, as they were isolated as pathogens of poikilothermic species. The strains from the study reported here have yet to be analysed for virulence. Although no animal model of diarrhoeal disease exists, a number of likely virulence factors can be assayed, including adhesion to and invasion of cultured epithelial cells. Comparison of the results of virulence assays performed on cells grown at different temperatures with the analysis of protein and LPS expression should enable the contribution of different cell surface components to be assessed.

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