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Internalization of *Aeromonas hydrophila* by fish epithelial cells can be inhibited with a tyrosine kinase inhibitor

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Abstract:
Aeromonas hydrophila is a Gram-negative bacterium that is pathogenic in fish, causing motile aeromonad septicaemia. It can enter (invade) fish cells, and survive as an intracellular parasite. The host-pathogen interaction and signal transduction pathway were studied by screening signal transduction inhibitors using carp epithelial cells and a virulent strain of the bacterium, PPD134/91. Genistein, a tyrosine kinase inhibitor, postponed internalization of *A. hydrophila* into host cells, suggesting that tyrosine phosphorylation plays a role in internalization. In contrast, staurosporine, a protein kinase C inhibitor, and sodium orthovanadate, a protein tyrosine phosphatase inhibitor, accelerated internalization of PPD134/91. Other virulent strains of *A. hydrophila* were also examined and it is likely that all strains, irrespective of serogroup, use the same signalling pathway to facilitate bacterial uptake.
Keywords: tyrosine kinase, *Aeromonas hydrophila,* fish epithelial cells
Abbreviation: EPC, epithelioma papillosum of carp.

Article:
INTRODUCTION
Aeromonas hydrophila is the causative agent of motile aeromonad septicaemia, found in a wide variety of freshwater fish species (Newman, 1993; Thune *et al.,* 1993). Motile aeromonad septicaemia outbreaks are common all over the world, and have been reported in Australia (Burke & Rogers, 1981), the south-eastern states of America (Pippy & Hare, 1969), Spain (Nieto *et al.,* 1985) and the Southeast Asia region (Roberts *et al.,* 1992). Outbreaks of motile aeromonad septicaemia usually occur only when the fish are immuno-compromised by stresses such as overcrowding or concurrent disease (Stevenson, 1988). A. *hydrophila* produces several virulence determinants, including cytotoxins and enterotoxins (Ljungh & Wadstrom, 1982; Yadav *et al.,* 1992) and a repertoire of enzymes that digest cellular components, mostly proteases and haemolysins (Allan & Stevenson, 1981; Leung & Stevenson, 1988). Other virulence factors such as the S layer (Dooley & Trust, 1988) and serum resistance (Mittal *et al.,* 1980; Leung *et al.,* 1995) are also implicated in aiding bacterial resistance to attack by the host's non-specific immune mechanisms.

A prerequisite for the initiation of infection is the initial adherence to, and invasion of, host epithelial cells. A. *hydrophila* is able to attach to collagen, fibronectin, serum proteins and glycoproteins found in fish mucus and epithelial cells (Atkinson & Trust, 1980; Ascencio *et al.,* 1991; Neves *et al.,* 1994). The putative adhesins thought to facilitate attachment may provide the necessary anchorage for the bacteria to facilitate subsequent invasion. We have recently studied the interaction between several different fish cell cultures and A. *hydrophila* and reported that *A. hydrophila* can enter into these cells and induce morphological changes (Leung *et al.,* 1996; Low *et al.,* 1998).

Invasion of cells by bacterial pathogens involves adherence of the organism to the host cell, followed by bacterial internalization into a membrane-bound vacuole inside the host cell (Moulder, 1985 ; Finlay & Falkow,

1989). Once inside the cell, these bacteria either remain within membrane-bound inclusions, as does *Salmonella typhimurium,* which multiplies efficiently within vacuoles (Garcia-del Portillo *et al.,* 1993), or escape into the cytoplasm, as does *Shigella flexneri,* which encodes enzymes to lyse the vacuolar membrane (Hale & Boventre, 1979).

Entry into non-phagocytic cells involves triggering host signal transduction mechanisms to induce rearrangements of the host cytoskeleton, thereby facilitating bacterial uptake (Finlay *et al.,* 1991 ; Pace *et al.,* 1993; Rosenshine & Finlay, 1993; Rosenshine *et al.,* 1996). Initiation of cytoskeletal rearrangement by enteropathogenic *Escherichia coli* appears to be triggered by activation of specific host tyrosine kinase activity. The induction of tyrosine kinase activity is mediated by the products of the enteropathogenic E. *coli cfm* genes. Enteropathogenic *E. coli* also causes an increase in the level of intracellular free calcium (Rosenshine *et al.,* 1992a). For *Y ersinia* species, invasin interacts with host integrins, causing the clustering of integrin into focal points, which possibly triggers host tyrosine kinase activity that may result in host cytoskeletal rearrangements (Young *et al.,* 1992). Thus, activation of tyrosine kinase appears to be a common feature in the signal transduction pathway of invasive pathogens leading to internalization.

The surface characteristics, virulence factors and distribution of *A. hydrophila* have been extensively studied. However, the interaction between the host and *A. hydrophila* remains largely unknown. In this work, we aimed to examine the interaction between *A. hydrophila* and carp epithelial cells. By using inhibitors of potential components of signal transduction pathways, it was hoped that the signalling pathway mediating internalization could be elucidated and thus that the pathogenesis of A. *hydrophila* would be better understood.

METHODS

Bacterial strains. Three virulent strains of *A. hydrophila* were used in this study (Leung *et al.,* 1995). *A. hydrophila* PPD134/91, PPD122/91 and PPD11/90 were isolated from diseased fish by the Primary Production Department of Singapore. They were tested using standard biochemical diagnostic kits (Microbact 24E System, Medvet Science; and BBL Crystal Enteric/Nonfermenter ID System, Becton Dickinson), and their identities were confirmed according to the criteria of Popoff (1984). Cultures were routinely grown in tryptic soy broth (TSB; Difco) or on tryptic soy agar (TSA; Difco) at 25 °C. Stock cultures were maintained at —80 °C as a suspension in TSB containing 25 % (v/v) glycerol.

Cell cultures. All tissue culture reagents were obtained from Gibco. EPC cells (epithelioma papillosum of carp, *Cyprinus carpio)* (Wolf & Mann, 1980) were grown in minimal essential medium (MEM) with Hanks' salts, 10 mM HEPES (pH 7.3), 2 mM glutamine, 023 $NaHCO_3$ and 10 % heat-inactivated foetal bovine serum at 25 °C in a 5 % CO, atmosphere. Cells were grown in 75 cm^2 flasks and split at least once a week by trypsin/EDTA treatment and dilution at 1 : 10 in fresh media.

Confocal microscopy. The Live/Dead BacLight Viability kit (Molecular Probes) was used to fluorescently label bacteria. NBD-phallacidin (Molecular Probes) was used to reveal the F-actin structure. EPC cells were seeded on glass coverslips and infected with A. *hydrophila* as described below. Coverslips were examined using a Nikon Optiphot microscope attached to the Bio-Rad MRC 500 confocal system (Lasersharp). Confocal images obtained under argon laser 488 nm blue excitation were photographed with a Polaroid Freeze-frame recorder using Kodak Tmax 100 film.

Signal transduction inhibitors. Inhibitors and their concentrations used are listed in Table 1. The solvent used to dissolve all chemicals was DMSO, except for sodium orthovanadate, which was dissolved in deionized water. Before use, the inhibitors were further diluted in MEM supplemented with 10 % (v/v) foetal bovine serum and this was added to the cultured EPC cells. The concentrations of inhibitors used were according to the manufacturers' recommendations. For genistein and staurosporine, concentrations used were those described by Rosenshine *et al.* (1992a).

Table 1. Classes of inhibitors used

Class	Specific inhibitor	Concn used (μM)	Time taken for infected EPC cells to reach stage II morphology (min)*
Control	–	–	58·3 ± 2·9
Protein tyrosine kinase inhibitors	Genistein	50, 100, 150, 200, 250†	96·7 ± 2·9‡
	Herbimycin A	10	58·3 ± 3·0
	Tyrphostin 47	50, 100†	60·7 ± 4·9
	Tyrphostin	50, 100	60·7 ± 4·9
Genistein analogue	Daidzein	150	61·7 ± 2·9
Protein tyrosine phosphatase inhibitor	Sodium orthovanadate	10	52·3 ± 2·9‡
Protein kinase C inhibitors	Staurosporine	0·005	50·0 ± 5·0‡
	Calphostin C	0·5	63·7 ± 8·1
Protein phosphatase inhibitors	Okadaic acid	0·1	61·5 ± 3·5
	Calyculin A	0·0001, 0·001, 0·002†	57·0 ± 1·4
Ca^{2+} channel blockers	Nifedipine	0·1, 1†	62·3 ± 6·6
	Verapamil HCl	1, 10†	58·7 ± 4·0

* Values shown are the mean ± SD from duplicate wells in three separate experiments.

† Concentrations used for the effect on infected EPC cells.

‡ Those inhibitors where the time taken to reach stage II was significantly different from the control ($P < 0·05$).

Effect of inhibitors on the morphological changes and viabilities in EPC and bacteria. Studies on morphological changes were conducted by seeding 3 x 10^5 fish cells into each well of a 24-well tissue culture plate (Falcon) and proceeding as described by Leung *et al.* (1996). Briefly, 5 ml stationary-phase cultures were prepared by inoculating TSB with *A. hydrophila* from frozen glycerol stocks and incubating overnight at 25 °C. Three hours before infection of cells, midexponential-phase cultures were prepared by diluting the bacteria 1:20 in fresh TSB and incubating at 25 °C for 2.5 h. Bacterial cells were pelleted and washed three times in PBS (137 mM NaCl, 2.7 mM Kl, 43 mM Na_2HPO_4 and 1·4 mM KH_2PO_4 at pH 72) before adding 5 μl to each tissue culture well (approximately 5 x 10^5 bacteria). After inoculation, the 24-well tissue culture plate was centrifuged (800 *g,* 5 min, 4 °C), then incubated for 30 min at 25 °C. Infected monolayers were washed once with MEM and then incubated in fresh, supplemented MEM. The morphology of EPC cells was examined every 5 min using an Axiovert 25CFL phase-contrast inverted microscope (Carl Zeiss) at x 200 magnification. In studies of different signal transduction inhibitors, EPC cells were incubated with the inhibitor for 30 min before bacterial infection. The time taken to reach each morphological stage was determined from duplicate wells in three separate experiments.

To investigate if the inhibitors could halt the morphological changes of previously infected EPC cells, the monolayers were first infected with bacteria for 5, 10, 15, 20, 30 and 45 min before the addition of inhibitors. To investigate if the effects of the inhibitors on EPC cells were reversible, the monolayers were incubated with the inhibitors for 30 min, and then washed twice with MEM to remove the inhibitors before infection with the bacteria.

To ensure that the inhibitors did not affect EPC cells, the cells were grown for 3 h in MEM containing genistein (250 μM), staurosporine (0·005 μM) or sodium orthovanadate (10 μM). A trypan blue exclusion assay was then used to determine whether treated EPC cells had similar viability to the untreated control. Similarly, *A. hydrophila* was treated with each of these three inhibitors for 3 h. Bacterial viability counts were determined before and after the 3 h incubation period. EPC and bacterial viabilities were determined by doing each experiment in triplicate.

Internalization assay. The invasion assay was as described by Leung & Finlay (1991) with minor modifications. Briefly, a monolayer of EPC cells was grown for 72 h in 24-well tissue culture plates to 100 % confluence. The cells were then washed with MEM and incubated with the respective inhibitors at 25 °C for 30 min before bacterial inoculation. The mixture was centrifuged for 5 min at 800 *g* at 4 °C and the plate was incubated for a further 30 min at 25 °C. The wells were washed once with MEM to remove bacteria remaining in the medium and incubated for 30 min with MEM containing gentamicin (100 μg ml^{-1}) to kill any residual extracellular bacteria. The EPC cells were washed three times with MEM to remove any remaining gentamicin that could kill bacteria subsequently released from cells. Triton X-100 (1 ml, 1%) was added to each well to lyse the EPC cells and dilution series were plated on TSA to determine the number of viable intracellular bacteria. The invasion rate was calculated as a mean of three trials in triplicate TSA plates.

Statistical analysis. All results from morphological and invasion assays were expressed as means ± SD. The data from these assays were analysed using one-way ANOVA followed by a Duncan multiple range test (SAS software). A value of $P < 0{\cdot}05$ was considered to be significant.

RESULTS

Morphological changes of EPC cells induced by A. hydrophila

Upon infection with the virulent *A. hydrophila* strain PPD134/91, EPC cells underwent a series of morphological changes empirically described as stages I—III. An uninfected monolayer of EPC cells appeared as a smooth sheet with the cells adhering tightly to their neighbours (Fig. la). In stage I (43 min post-infection), the cells became slightly detached from one another (Fig. 1b). The smooth appearance was lost and the cells appeared darker. In stage II (58 min post-infection), the separation between cells became more apparent, large holes separated cells (about 50 % of the cells remained attached to the tissue culture plate) and they were elongated to form long spindles (Fig. 1c). In stage III (about 90 min post-infection), the cells became rounded and the spindle connections were lost (Fig. 1d). Bacteria enclosed in a vacuole could be seen in the cells but no other cellular organelle was visible (Fig. 1g).

Effects of A. hydrophila on F-actin distribution

The distribution of microfilaments (F-actin) was also examined during *A. hydrophila* infection. In the control EPC cells, F-actin was arranged in a network and stress fibres were seen (Fig. 2a). Cells that were infected with PPD134/91 had localization of F-actin (actin clouds) (Fig. 2b). These actin clouds were observed in stages I and II but not in stage III of infected cells (data not shown).

Screening of signal transduction inhibitors

Different classes of inhibitors were screened to better understand the signal transduction pathway used by *A. hydrophila* when entering EPC cells. The times taken to reach the respective stages were recorded for each inhibitor. Stage II was chosen as an end point in scoring because of the clarity of the morphological changes. Among all the inhibitors used for screening, only genistein, a tyrosine kinase inhibitor, delayed the time taken for induction of morphological changes in EPC cells (Fig. if), whereas staurosporine, a protein kinase C inhibitor, and sodium orthovanadate, a protein tyrosine phosphatase inhibitor, decreased the time taken for changes to occur (Table 1). Staurosporine accelerated the morphological changes of EPC cells to a greater extent than sodium orthovanadate (Table 1 and Fig. le). Inhibitors alone did not induce any morphological changes in control EPC cells.

Concentration effects of genistein

The effect of increasing concentrations of genistein on internalization of *A. hydrophila* was examined by measuring the time taken for the EPC cells to reach stage I and stage II morphology (Fig. 3) and by measuring the number of bacteria internalized (Fig. 4). With increasing concentrations of genistein, the time taken for EPC cells to reach stage II morphology was significantly increased ($P < 0{\cdot}05$) (Figs if and 4). Genistein (50 μM) had negligible effect on the morphological effects of PPD134/ 91 on EPC cells. However, with increasing concentrations of genistein from 100 μM to 250 μM, times taken to reach stages I and II increased significantly (Fig. 3). In the presence of 250 μM genistein, infected EPC cells took 30 min longer than the control cells to reach

stage I morphology and 41 min longer to reach stage II morphology. The time interval between the start of stage I and the start of stage II was not significantly affected by the increasing concentrations of genistein ($P > 0.05$).

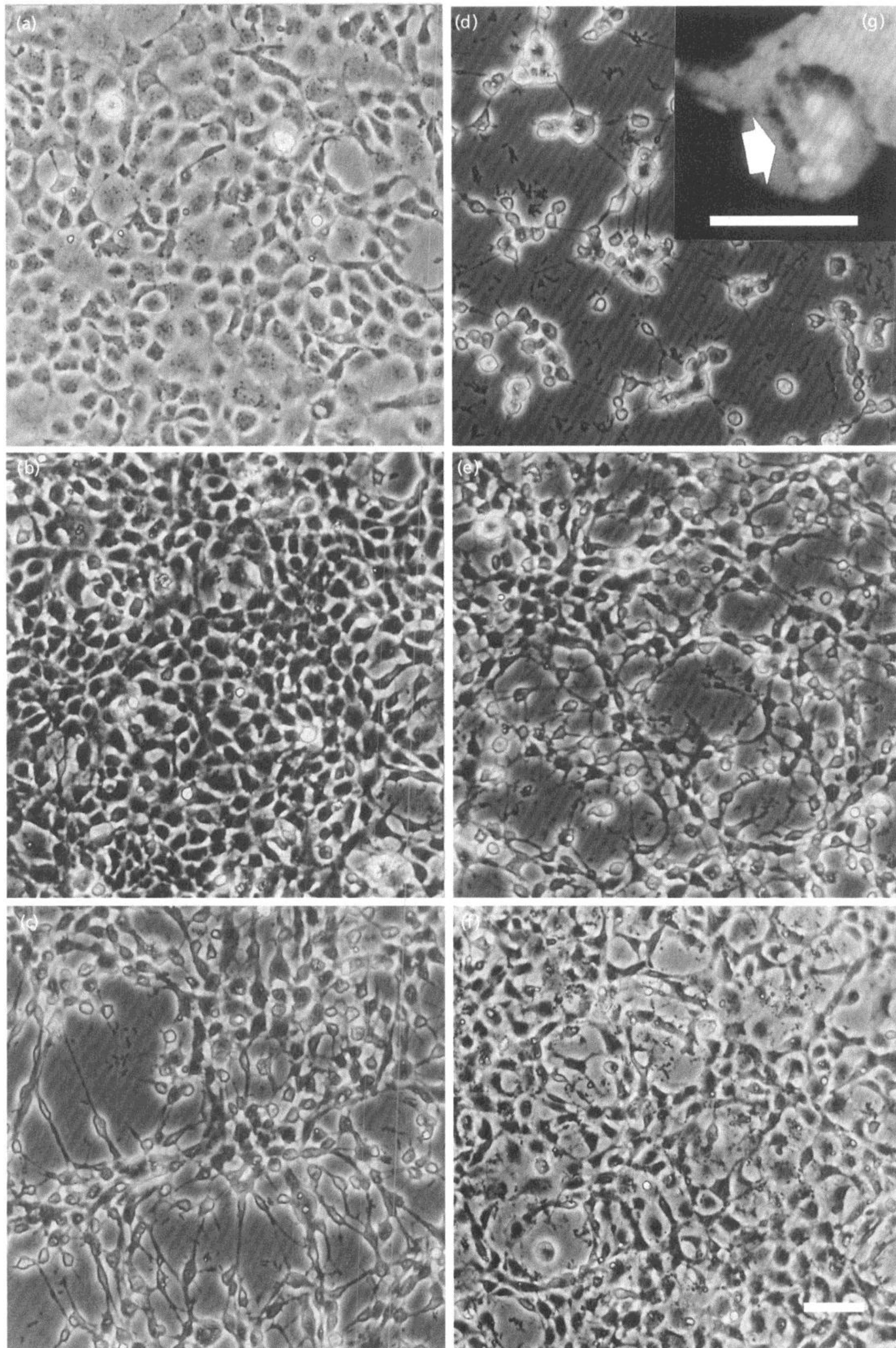

Fig. 1. Phase-contrast and confocal micrographs of EPC cells infected with *A. hydrophila* PPD134/91. Different stages of infection and treatments are included. (a) Uninfected control; (b) stage I of infection (43 min); (c) stage II of infection (58 min); (d, g) stage III of infection (70 min); (e) 43 min with 0·005 μM staurosporine; and (f) 58 min with 250 μM genistein. In (g) *A. hydrophila* was stained using the Live/Dead BacLight Viability kit. Bacteria were seen inside the distorted and ghost-like EPC cells as indicated by the arrow in (g). Bar in (f) (also applies to a–e), 15 μm; bar in (g), 10 μm.

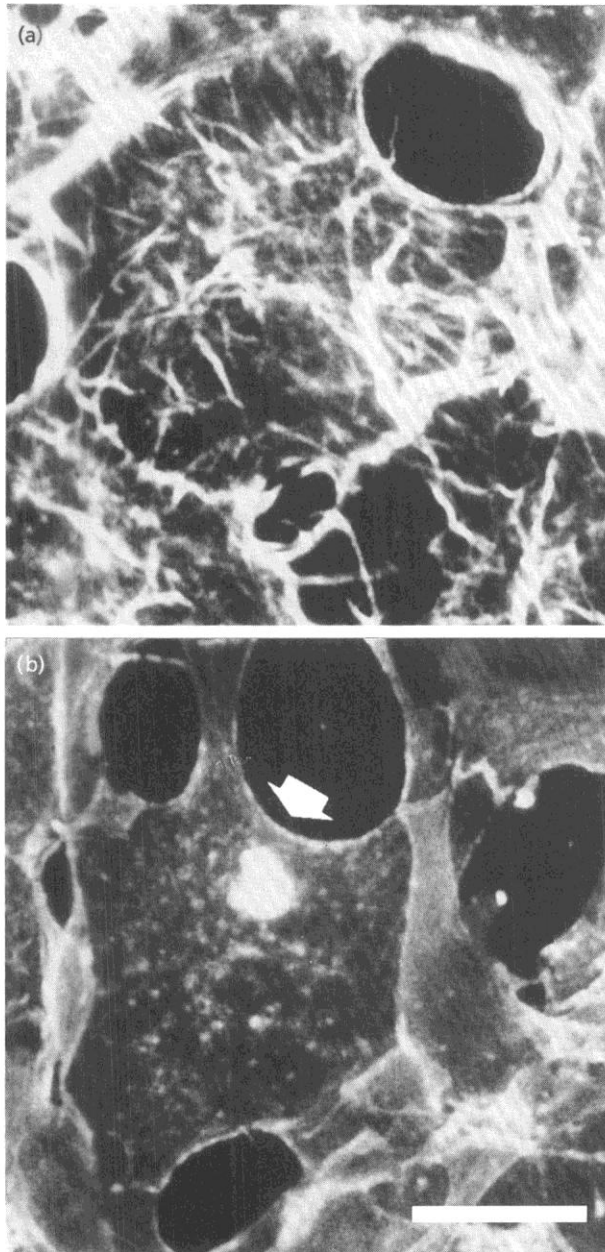

Fig. 2. F-actin-stained confocal micrographs of EPC cells infected with *A. hydrophila* PPD134/91. (a) Uninfected control; (b) stage I of infection. In the control EPC cells, F-actin was arranged in a network and stress fibres were seen. Actin clouds were visible during stage I of infection (marked by white arrow). Bar, 5 μm.

The number of bacteria internalized in the presence of different concentrations of genistein was compared to an untreated control, which was taken to be 100%. Similar concentration-dependent inhibitory effects of genistein were observed in this invasion assay as in the morphological studies. The number of intracellular bacteria decreased with increasing concentrations of genistein. At 50 μM genistein there was no apparent effect on the number of internalized bacteria, whereas at 250 μM genistein the number of bacteria internalized were reduced to 23% of the control cultures (Fig. 4). For all subsequent experiments, morphological assays (time taken to reach stage II) were used to study the effects of inhibitors on the interaction between PPD134/91 and EPC cells as they were easier to perform and correlated well with the internalization assay.

The ability of genistein to delay the effects of PPD134/91 on EPC cells that were already infected with bacteria was investigated by adding different concentrations of genistein at 5, 10, 15, 20, 30 and 45 min after infection of cells (Table 2). It appeared that morphological changes could be delayed by genistein for the first 20 min after infection. If applied 20 min after infection, only the higher concentrations of genistein (250 and 200 µM) significantly (*P* < 0·05) delayed morphological changes (Table 2). If applied 30 min after infection, even the highest concentration of genistein (250 µM) failed to delay the effects of PPD134/91 on EPC cells.

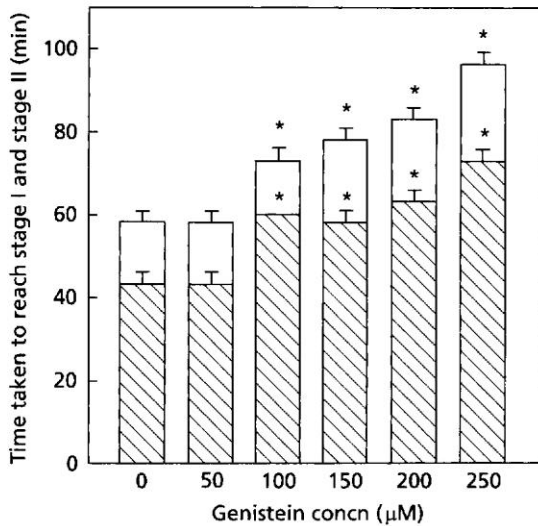

Fig. 3. Effect of different concentrations of genistein on morphological changes of EPC cells induced by *A. hydrophila* PPD134/91. Stage I (hatched bars) was defined as the time when the EPC cells became slightly detached from one another. The smooth appearance was lost and the cells appeared darker in colour. Stage II (open bars) was defined as the time when larger holes separate infected cells and about 50% of the cells remained on the tissue culture plate. The infected EPC cells were elongated to form long spindles. Values shown are the mean ±SD from duplicate wells in three separate experiments for each sample. Those concentrations where the time taken to reach stage II was significantly different from that in control cultures (*P* < 0·05) are marked with asterisks.

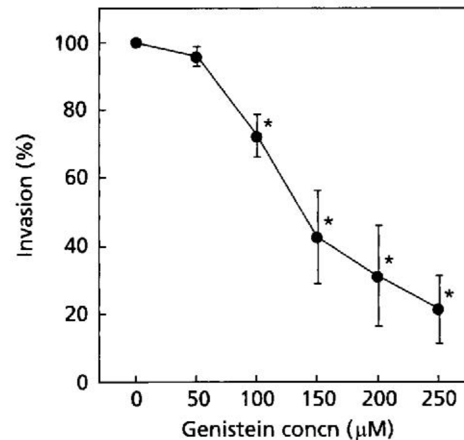

Fig. 4. Effect of different concentrations of genistein on invasion rate in EPC cells. Values shown are the mean ± SD of three experiments in triplicate TSA plates for each sample. Those concentrations where the invasion rate was significantly different from that in control cultures (*P* < 0·05) are marked with asterisks.

Table 2. Effect of addition of genistein at various time points after infection with *A. hydrophila* PPD134/91

Values shown are the mean ± SD from duplicate wells in three separate experiments.

Genistein concn (µM)	Time to reach stage II when genistein added after:					
	5 min	10 min	15 min	20 min	30 min	45 min
0	58·3 ± 2·9	58·3 ± 2·9	58·3 ± 2·9	61·7 ± 7·6	61·7 ± 7·6	55·0 ± 5·0
50	58·3 ± 2·9	60·0	58·3 ± 2·9	61·7 ± 7·6	61·7 ± 7·6	55·0 ± 5·0
100	70·0 ± 5·0*	66·7 ± 2·9	61·7 ± 2·9	65·0 ± 5·0	61·7 ± 7·6	55·0 ± 5·0
150	75·0 ± 5·0*	70·0 ± 5·0*	65·0 ± 5·0*	70·0 ± 5·0	68·3 ± 7·6	55·0 ± 5·0
200	80·0 ± 5·0*	76·7 ± 7·6*	71·7 ± 2·9*	75·0 ± 5·0*	68·3 ± 7·6	55·0 ± 5·0
250	85·0 ± 5·0*	80·0 ± 9·0*	76·7 ± 2·9*	78·3 ± 2·9*	71·7 ± 7·6	55·0 ± 5·0

* Those concentrations where the time taken to reach stage II was significantly different from that in control cultures (*P* < 0·05).

Effects of inhibitors on the functions of PPD134/91 and EPC cells

The viability of *A. hydrophila* PPD134/91 treated with genistein (250 µM), staurosporine (0.005 µM) or sodium orthovanadate (10 µM) in MEM for 3 h was 96·9 ± 3.3 %, 94·5 ± 6·4% or 104·1 ± 7·9% (*n* = 3) of untreated controls, respectively. Hence genistein did not interfere with bacterial viability. The viability of EPC cells

treated with genistein (250 µM), staurosporine (0·005 µM) or sodium orthovanadate (10 µM) in MEM for 3 h was 100 % (n = 3) of untreated controls. Thus, genistein does not interfere with the viability of EPC cells.

To determine whether genistein permanently reduced the ability of *A. hydrophila* to induce changes in EPC cells, the bacteria were incubated with 250 µM genistein for 30 min, washed and used to infect EPC cells. The time taken to reach stage II morphology (57 + 2 min) was similar to that seen with the untreated bacteria (58 ± 3 min). Hence genistein did not impair the ability of *A. hydrophila* to induce morphological changes. To determine whether genistein permanently affected host cells, EPC cells were incubated with 250 µM genistein for 30 min. After the genistein was removed by washing, PPD134/91 induced stage II morphology in treated EPC cells at the same time (58 ± 1 min) as untreated controls (58 ± 3 min). Hence the effects of genistein on EPC cells are reversible. Similar results were found for staurosporine and sodium orthovanadate (data not shown).

Effects of daidzein on internalization

Daidzein is an inactive analogue of genistein, and does not inhibit the activity of tyrosine kinase. To confirm that genistein specifically inhibited the action of tyrosine kinase, the effect of daidzein on the time taken to reach stage II was examined. It was found that daidzein had no significant effect on the morphological changes in EPC cells induced by *A. hydrophila* (Table 1).

Effects of inhibitors on other virulent strains

The effects of the different signal transduction inhibitors on two other virulent strains of *A. hydrophila,* PPD122/ 91 and PPD11/90, were also examined. Genistein significantly delayed the time taken for induction of morphological changes in the EPC cells by either PPD122/91 or PPD11/90. In contrast, staurosporine decreased the time taken for the changes to occur (Table 3).

Table 3. Effects of inhibitors on morphological changes of EPC induced by other virulent strains of *A. hydrophila*

Inhibitor	Time taken to reach stage II (min)*	
	Strain PPD122/91	Strain PPD11/90
Control	93·3 ± 2·9	51·7 ± 2·9
Genistein, 50 µM	93·3 ± 2·9	51·7 ± 2·9
Genistein, 100 µM	103·3 ± 5·8†	61·7 ± 10·4†
Genistein, 150 µM	105·0 ± 5·0†	71·7 ± 2·9†
Genistein, 200 µM	106·7 ± 2·9†	76·7 ± 2·9†
Genistein, 250 µM	110·0†	83·8 ± 2·9†
Staurosporine, 5 nM	81·7 ± 2·9†	45·0†

* Values shown are the mean ± SD from duplicate wells in three separate experiments.

† Those inhibitors where the time taken to reach stage II was significantly different from the control ($P < 0·05$).

DISCUSSION

Genistein was found to inhibit the rate of morphological changes induced by *A. hydrophila,* whereas staurosporine and sodium orthovanadate accelerated it (Table 1). Genistein is a specific inhibitor of tyrosine kinase, with negligible effect on the activity of serine and threonine kinases (Akiyama *et al.,* 1987), whereas sodium orthovanadate is an inhibitor of protein tyrosine phosphatase (Leis & Kaplan, 1982). Because the effects of genistein and sodium orthovanadate are consistent with their antagonistic enzymic actions, it is likely that tyrosine kinase is involved in interactions between A. *hydrophila* and EPC cells. Staurosporine is known to inhibit protein kinase C *in vitro* and increases tyrosine phosphorylation in PC12 cells (Rasouly & Lazarovici, 1994). Therefore, the accelerated rate of morphological change produced by staurosporine might be associated with enhanced tyrosine phosphorylation. Verapamil and nifedipine are Ca^{2+} channel blockers (Varadi *et al.,* 1995). Our data show that both verapamil and nifedipine failed to block the morphological changes induced by PPD134/91 in EPC cells, suggesting that the internalization process is not mediated by Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Likewise, calphostin C, a potent protein kinase C inhibitor (Kobayashi *et al.,*

1989), and okadaic acid and calyculin A, inhibitors of protein phosphatase (Ishihara *et al.,* 1989), did not alter the morphological changes induced by PPD134/91, indicating that the protein kinase C pathway of signal transduction might not be involved in the bacterial internalization process.

On the basis of these results it appears that a tyrosine kinase is involved in the signalling pathway between *A. hydrophila* and the EPC cells. Genistein also inhibits bacterial invasion in *Listeria monocytogenes* (Tang *et al.,* 1994) and enteropathogenic *E. coli* (Rosenshine *et al.,* 1992b). Genistein, staurosporine and sodium orthovanadate could affect internalization by other nonselective mechanisms, including affecting bacterial viability or some bacterial function needed for internalization or the functions of EPC cells. Each of these possibilities was examined and the data show that these inhibitors did not affect the bacteria and the EPC cells in a non-specific manner. Thus, these inhibitors appear to act primarily by specifically affecting bacterial internalization.

Genistein was able to reduce morphological changes for up to 20 min after infection. Twenty minutes may be the time required for signal transduction initiated by tyrosine kinase to transmit to downstream signalling molecules and once this has been achieved the bacterial internalization process is irreversible (Table 2). The time taken from stage I to stage II was not significantly different ($P > 0{\cdot}05$) with increasing concentrations of genistein (Fig. 3). This suggests that on reaching a stage I morphology (43 min for control cells), the signal generated by tyrosine kinase has already been transduced to a downstream level that cannot be blocked by a tyrosine kinase inhibitor, such as genistein.

Daidzein is an inactive analogue of genistein that does not inhibit the action of tyrosine kinase. We found that daidzein had no significant effect on the invasion rate of *A. hydrophila* on EPC cells. This further supports the hypothesis that genistein blocks *A. hydrophila* internalization specifically by tyrosine kinase inhibition.

Signal transduction pathway of A. hydrophila

Our results suggest that there is a signal transduction pathway participating in the interaction between the pathogen *A. hydrophila* and EPC cells. It is likely that *A. hydrophila* adheres to the host cell surface before internalization. The adhesins may be the 0-antigen of lipopolysaccharide (Merino *et al.,* 1996) or the outer-membrane protein isolated by Lee *et al.* (1997) which mediate the adhesion and internalization of the bacterium. At present it is not clear whether *A. hydrophila* also produces invasin(s) to aid in internalization.

After adhesion, *A. hydrophila* probably initiates a signalling cascade that involves tyrosine kinase. Hence this kinase appears to function as part of an uptake signal for this fish pathogen. At the end of the signalling pathway, *A. hydrophila* causes the rearrangement of microfilament (F-actin) in EPC cells to form actin clouds (Fig. 2). These actin clouds might be associated with the internalization of bacteria. Subsequently, the internalized bacteria replicate intracellularly and cause morphological changes in the EPC monolayer.

Our previous report suggested that PPD134/91, PPD122/91 and PPD11/90 were the three major serogroups among our Southeast Asia isolates of *A. hydrophila* (Leung *et al.,* 1995). By screening the same repertoire of signal transduction inhibitors, we have found that a common signal transduction pathway was shared by all serogroups (Table 3). *A. hydrophila* is believed to be a very heterogeneous species with different surface characteristics (Mittal *et al.,* 1980), which presents a potential problem for vaccine development (Stevenson, 1988). If *A. hydrophila* uses a common invasion and internalization pathway, new approaches could be designed to specifically block this common signal transduction process for use in treatment and prophylaxis.

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