Influence of temperature and nutrients on R+ plasmid conjugation transfer from an environmental *E. coli* strain

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The increase in the number of bacteria capable of transfer resistance plasmids, detected in aquatic systems, may be due not only to an increase in dumpings, but also to the fact that in these ecosystems processes of plasmid transfer actively occur. Mc Nichol *et al.* (1982) suggested that the latter may be the origin of the formation of plasmid pools.Toranzo *et al.* (1984) reported the possibility that the pathogen microorganims may participate in *in situ* conjugations processes in aquatic systems. Scanferlato *et al.* (1989) remarked the survival of G.E.M.s in these systems.

The incidence of these facts on public health requires that more detailed studies be done on those systems which are potentially suitable for plasmid transfer by conjugation. Because *in situ* experiments are subjet to numerous environmental factors, not always predictable nor controlable, *in vitro* experimental models are necessary, despite their multiple limitations.

The aim of our first experiments was, therefore, to determine the ambient factors which may limit plasmid transfer by conjugation in aquatic systems.

From the freshwater system isolations, we chose as donor a plasmid containing *E.coli* strain, R+ to ampicillin and gentamicin. As the recipient strain we used a non plasmid containing *E.coli* K12, J62, chromosomic resistant to nalidixic acid. As inoculum we used 10^8 u.f.c. of donor and 0,5 10^8 u.f.c. of recipient strain, in 5ml of mating medium. Transconjugants were selected and counted on Mc Conkey agar (OXOID) plates, suplemented with ampicillin (64 µg/ml) and nalidixic acid (32 µg/ml).

In order to determine the influence of the river temperature, mating experiments were carried out in T.S.B. broth (OXOID) during 2 hours, at 20°C (mean river temperature), and that the control 37°C. The transfer frequencies obtained both at 20°C and 37°C were of the order of 10^{-3} (n° of transconjugants /n° of initial donors). These results suggest that the river temperature is not a limiting factor for transfer by conjugation.

To determine the minimun nutritional requirements, mating experiments were done on T.S.B. and on a series of decreasing T.S.B. concentrations and finally in the absence of nutrients (autoclaved distilled water). The inocula were obtained in standard conditions and later washed 3 times with P.S.B.(pH 7,2). Our first results show a decrease in transfer frequency, parallel to the decrease in nutrient concentration, although the frequency values obtained never were below 10^{-6}. We must highlight that transfer frequency values are obtained even in the absence of nutrients.

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Virulence factors of environmental strins of *Aeromonas hydrophila*

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In recent years, the high development of the aquaculture of fish and shellfish has originated an increase of the problems related to the infections of these animals by different pathogenic microorganisms.

The infective capability of the pathogenic microorganisms is generally related to the presence of virulence factors. These virulence factors seem to make possible the attachment of bacteria to host cells and enterotoxin synthesis by increasing the invasive capability of the strains [4]. One of the most important factors involved in the virulence of the pathogenic strains consists of an efficient iron-sequestering system mediated by siderophores [2,6], which allows the bacteria to grow in the iron-limiting conditions imposed by the high-affinity iron-binding proteins present in the organic fluids [7].

In this study the presence of haemolytic activity and the production of diffusible siderophores by *A. hydrophila* strains isolated from water environments and animals (shellfish and fish) were analyzed.

ß-haemolytic isolates were identified on blood agar plates consisting of Blood Agar Base (Difco) and 5% washed sheep erythrocytes. The haemolytic activity was recorded like clearance of the medium around the growth zone after 24 h at 25°C.

A. hydrophila strains were cultured in M9 minimal medium supplemented with 0.2% (w/v) Casamino Acids (Difco). The iron chelator ethylenendiamine-di-(o-hydroxyphenyl acetic acid) (EDDA) was added at a concentration of 10 µM to achieve the iron limitation conditions.

The production of siderophore compounds by the strains was tested on blue agar plates as described by Schwyn & Neilands [9]. The method is based on the fact that the dye chrome-azurol S (Sigma) incorporated into the medium, can form stable complexes with iron. Therefore, when a strain is able to produce a diffusible siderophore (which removes the iron from the complex) the colour turns to yellow-orange around the colony after 24-48 h at 25°C.

The ability of the strains of *A. hydrophila* to grow under iron-limiting conditions and the haemolytic activity were tested to verify their role as putative virulence factors and to demonstrate the relationship with the plasmid content of the strains.

More than 75% of the strains tested gave an orange halo between 6-8 mm after 48 h, and less than 10% of the strains were negative in producing siderophore activity. Similarly, the majority of the strains (more than 90%) tested showed haemolysis activity. A higher number of isolates that showed siderophore and total haemolysis activities were obtained from seawater and animals in comparison with the number of strains from freshwater environment (23.3%, and 11.7% vs 6.7%, respectively).

The relationship between siderophore production and plasmid content was strong, since more than 74% of the strains with siderophore possessed plasmids, although the percentage varied depending on the source of strain (between 85% for freshwater strains and 68% for seawater strains). In the case of the haemolytic activity the percentage of strains with this characteristic which harbor plasmids is lower than for siderophore (about 60%).

The relationship between the presence of plasmids and some pathogenic characteristics of the strains, such as enterotoxigenicity, haemolysin production or presence of surface antigens have been reported in human pathogens, mainly *Enterobacteriaceae* [4,5,8,12]. However, for other species, correlation between virulence factors and plasmid profiles have only been reported in some instances. From the results obtained in this study, it may be concluded that there is a close relationship between the presence of plasmid and the siderophore production, since more than 70% of the strains with siderophores also possessed plasmids. This conclusion is supported by the curing experiments where the plasmidless derivatives showed a loss of the ability to grow in iron-limiting conditions. These results are in agreement with those reported by Crosa [1], Crosa et al. [3] and Walter et al. [11] for *Vibrio anguillarum* strains. However, about 30% of the strains analysed retained the iron-sequestering system after loss of plasmids. These results indicate that the genes coding for this system in *A. hydrophila* are located in both plasmid and chromosome. Similar conclusions were obtained by Valvano et al. [10] studying the aerobactin iron transport system in human invasive strains of *Escherichia coli* K1, and Lemos et al. [7] in anguibactin-mediated system of *V. anguillarum* strains.

In the present study, more than 90% of the strains studied had a haemolytic activity being higher in the strains isolated from animals (100%) than in those isolated from water environments (about 80%). This property seems to be chromosome-coded since plasmidless strains maintained the haemolytic activity. The role of the haemolysins in the pathogenicity of *A. hydrophila* is difficult to establish. In our opinion, the haemolysins could increase the availability of iron by the microorganisms by mean of the erythrocyte lysis, and in this way, there must be a close correlation between haemolysin production and the iron-using system.

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