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Full Length Research Paper

Pasteurella multocida X73 Able to Develop Natural Competence in Presence of Hyaluronidase-Producing *Staphylococcus aureus* Strain

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Bacteria presenting hyaluronic acid-rich capsules were thought to be unable to develop natural competence and to uptake exogenous DNA. In this article, genetic transformation of *Pasteurella multocida* X73 is achieved with the aid of physiological concentrations of calcium ions. The encapsulated *Pasteurella multocida* X73 was cultivated as a mixed culture in proximity to a hyaluronidase-producing strain of *S. aureus*. The *Pasteurella-Escherichia coli* shuttle vector pPBA1100 is investigated to test the competence ability of *P. multocida* X73 under natural conditions using bottled mineral water, which contained between 0 and 11 mM Ca^{2+}. It was found that transformation frequencies with *P. multocida* X73 were similar to those reported for other gram-negative bacteria known to develop natural competence. This result will contribute to the understanding of how bacterial horizontal gene transfer is happening in natural ecosystems. Other studies should be carried on other Bacteria presenting hyaluronic acid-rich capsules and hyaluronidase-producing strains.

Key words: Natural competence, *Pasteurella multocida* X73, *Staphylococcus aureus*

INTRODUCTION

The gram negative bacterium *P. multocida* is a heterogeneous species, which exhibits a broad host range upon most mammals, birds and even human being. It can cause specific diseases such as hemorrhagic septicemia and fowl cholera. It is also a major cause in contributing in diseases such as the respiratory tract of different animals (Adlam and Rutter, 1989). *P. multocida* can be capsulated or non capsulated.

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The capsulated strain can be separated into five serological groups A, B, D, E, or F (Carter, 1967; Rimler and Rhoades, 1987). The X73 strain belongs to the serological group A (A: 1) with a high amount of hyaluronic acid and confers a highly mucoid colony morphology (Pandit and Smith, 1993). A hyaluronic acid consists of a polymer of N-acetylglucosamine and glucuronic acid probably arranged as alternating units in a flexible chain, which can be depolymerized or hydrolyzed in presence of the mucolytic enzyme Hyaluronidase into N-acetylglucosamine. Hyaluronidases are produced by a variety

of organisms and can be divided into three categories (Kreil, 1995):

1- The testicular type hyaluronic acid-4-glycanohydrolases.
2- The hyaluronic acid-3-glycanohydrolases produced by leeches and hookworms.
3- The hyaluronic acid lyases or bacterial Hyaluronidases.

Hyaluronidase-producing strain of *Staphylococcus aureus* is one of those bacterial organisms implicated in infections (Adlam and Rutter, 1989).

In 1989, Bredy and Botzler reported that Sodium, protein, calcium, and magnesium have been associated with both the survival of *P. multocida* and with avian cholera mortality.

On the other side, it was shown that increased calcium and magnesium levels have been associated with persistence of *P. multocida* and avian cholera mortality (Windingstad et al., 1984; Windingstad et al., 1988; Price et al., 1992). In addition, it was demonstrated that *P. multocida* has been isolated from the water and sediment of wetlands experiencing avian cholera epizootics, and the bacteria can persist in wetland soil and water (Rosen and Bischoff, 1949; Backstrand and Botzler, 1986; Rosen, 1969; Korschgen et al., 1978; Price and Brand, 1984; Ausubel et al., 1987; Samuel et al., 2003).

Horizontal gene transfer's studies between bacteria in natural aquatic systems have shown that the occurrence takes place by conjugation (O'Morchoe et al., 1988), transduction (Saye et al., 1987), transformation (Paul et al., 1991; Stewart, 1992; Stewart and Sinigalliano, 1990) and cell contact-mediated transformation (Price and Brand, 1984).

It was reported that, in gram-positive bacteria, competence is usually induced and controlled by competence factors which are secreted into the medium (Saunders and Saunders, 1988). In such cases, the competence is induced as soon as the competence factor has reached a certain concentration (Stewart and Carlson, 1986). In contrast, in gram-negative bacteria, competence is usually internally regulated (Paget and Simonet, 1994; Stewart and Carlson, 1986). The problem with studies of natural competence development is finding the relevant environmental parameters which trigger this induction. In 1994 Lorenz and Wackernagel have reviewed the known parameters. The bacteria have to be metabolically active, and a shift to unbalanced growth, e.g., by nutrient limitation, can trigger competence development in many gram-negative bacteria. The multiple variations in the required conditions found illustrate the different ecologies of the variety of bacteria chosen. For example, *Azotobacter vinelandii*, a typical soil bacterium, is best transformable after growth in minimal media (Page and Sadoff, 1976) while *Acinetobacter calcoaceticus*, a ubiquitous human pathogen (opportunist) usually found on the skin develops competence in complex as well as minimal media (Palmen et al., 1993).

It was shown that incubation of *E. coli* cells with small temperature shifts (5 or 10°C) and even in the absence of temperature variations achieved the highest transformation frequencies which could be obtained under environmental conditions (Baur et al., 1996). *E. coli* can develop genetic competence under environmental conditions when in contact with surface water originating from calcareous regions. Calcium concentrations above 1 mM, which are

often found in spring water and river water, are sufficient to make gram-positive and gram-negative bacteria competent, and no additional competence-promoting factors or buffering substances are needed. (Trombe, 1993. Baur et al., 1996).

In 1975 Carter and Rundell developed a simple test in which *P. multocida* X73 (serogroup A) strains are recognized by depolymerization of the capsule after growth in proximity to a hyaluronidase-producing strain of *S. aureus*. On the basis of their findings and the author's one (Abdelhak, 2009), this study investigates for the first time, how the bacterial horizontal gene transfer is happening in natural ecosystems for those presenting hyaluronic acid-rich capsules.

MATERIALS AND METHODS

Bacteria, plasmid, media and growth conditions

Bacterial strains and plasmids used in this study are shown in Table 1. The encapsulated *P. multocida* X73 and a hyaluronidase-producing strain of *S. aureus* were separately cultivated over night at 37°C in brain heart infusion (BHI) (Oxoid, Hampshire, England).
The mineral water samples were sterilized by filtration through nitrocellulose membrane filters (0.22-mm pore size). The mineral water samples used and their calcium contents are presented in Table 2.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
P. multocida X-73	Serotype A:1 wild-type strain	Institute of Microbiology and Epizootics, FU-Berlin, Germany
S. aureus	Hyaluronidase-positive	Institute of Microbiology and Epizootics, FU-Berlin, Germany
S. aureus	Catalase-negative, Hyaluronidase-negative	Department of infectious diseases, Centre hospitalier universitaire de Batna, Algeria
Plasmids		
pPBA1101	*E. coli-P. multocida* shuttle vector, Kan^r	Homchampa et al., 1997

Oligonucleotides

Two antisense (M13 universal primer and M13 universal reverse primer) primers were custom synthesized, they had following sequences: Oligonucleotide pba-1, 5′-ACTGGCCGT CGTTT TAC-3′, and pba-2, 5′-GTCATAGCTGTTTCCTG-3′. Both of them are sitting in inverse position on the pPBA1100 and were qualified to amplify the whole pPBA1100.

Natural transformation protocol

P. multocida X73 and *S. aureus* were grown separately in brain heart infusion (BHI)(Oxoid, Hampshire, England) overnight at 37°C. The overnight cultures were used to inoculate fresh culture which was grown at 37°C to an optical density at 600 nm of 0.6. The cells were centrifuged at 10,000 x *g* for 10 min, washed very gently twice with ice cold

sterile mineral water sampled and finally separately resuspended in 40µl aliquots.
1 µl from each aliquot were then mixed in 38µl sterile water and 2.5 ng of the *Pasteurella-Escherichia coli* shuttle vector pPBA1100 were then added. The samples were then shuttled gently for 10 days at 4°C. Immediately after transformation, 1 ml SOC medium was added to the different samples and incubated at 37°C for two hours. Finally the cultures were plated overnight by 37°C on Columbia agar plates (Oxoid, Hampshire, UK) containing 5% sheep blood supplemented with 50 µg/ml of kanamycine (Kan). Control experiments are presented in table 2.

Plasmid isolation and digestion
The pPBA1100 was prepared and digested with the restriction endonuclease *Eco*RI (Life Technologies, UK) as described previously (Ausubel et al., 1987). Endonuclease-digested DNA was electrophoresed through a 0.7% agarose gel.

Polymerase chain reaction (PCR)
PCR amplification was performed directly from single colonies grown on sheep blood agar plates. A pipette tip was lightly touched onto a colony. This sample was then resuspended in PCR amplification mixture. DNA amplification was performed with the Expand high-fidelity PCR kit, using the reaction conditions specified by the manufacturer (Roche Molecular Biochemicals) and carried out using a thermal programmer (Gene Amp PCR System 2400; Perkin-Elmer Cetus Inc., Norwalk, Conn.). PCR-amplified DNA was then electrophoresed through a 0.7% agarose gel.

RESULTS

The encapsulated *P. mutocida* X73 is truly able to develop natural competence in presence of hyaluronidase-producing *Staphylococcus aureus* strain.
Table 2, gives the number of transformants obtained when *P. multocida* X73 cells were incubated in natural water of known Ca^{2+} concentrations. Bidistilled water alone gave no transformants at all. Incubation of *P. multocida* with pure Hyaluronidase (Roche Molecular Biochemicals) in Bottled mineral water increases the number of transformants in contrast to native hyaluronidase produced by *Staphylococcus aureus*.

Table 2. Ca^{2+} concentrations of water samples and related numbers of transformants.

Sample type or source	Sample no.	[Ca^{2+}] (mg/l)	No. of transformants/ml
Bottled mineral water (Algeria)			
Hayet (Danone) + *S. aureus* Hyaluronidase-positive	1	120	576
Vie pure (Nestlé) + *S. aureus* Hyaluronidase-positive	2	57.9	322
Ifri + *S. aureus* Hyaluronidase-positive	3	81	208
Control solutions			
Bidistilled water	4	0.0	0
Bidistilled water + *S. aureus* Hyaluronidase-negative	5	0.0	0
Bidistilled water + *S. aureus* Hyaluronidase-positive	6	0.0	0
Bidistilled water + Pure Hyaluronidase	7	0.0	0
Hayet (Danone) + Pure Hyaluronidase	8	120	2211
Vie pure (Nestlé) + Pure Hyaluronidase	9	57.9	1122
Ifri + Pure Hyaluronidase	10	81	1812
Bottled mineral water Hayet (Danone)	11	120	0
Bottled mineral water Vie pure (Nestlé)	12	57.9	0
Bottled mineral water Ifri	13	81	0

The titration experiments were undertaken using different concentration of the mentioned enzyme and different concentrations of the hyaluronidase-producing *Staphylococcus aureus* strain. The maximal transformation efficiency was obtained at 1.85U/ml for the pure enzyme and 1µl for the native one produced by *Staphylococcus aureus* strain (data no shown).

The 2.5 ng DNA concentration was largely sufficient to obtain recombinants. Recombinants *P. multocida* X73 were checked using *Eco*RI endonuclease (Fig. 1) and PCR (Fig. 2).

We have used a small plasmid to obtain maximal transformation frequencies because it has been demonstrated that the transformation frequencies obtained are inversely proportional to the size of the transforming plasmid (Hanahan, 1983; Inoue et al., 1990).

No recombinants *P. multocida* X73 were detected by the control experiment involving a non-hyaluronidase-producing strain *S. aureus* substantiating the effect of the staphylococcal enzyme.

DISCUSSION

The hyaluronic acid-rich capsules make affected bacteria relatively retractile to transformation. For the first time, this study demonstrates that encapsulated bacteria are also able to develop natural competence in presence of those producing hyaluronidase.

Natural genetic transformation in this work was only performed on *Pasteurella multocida* X73 using a hyaluronidase-producing *Staphylococcus aureus* strain. Other researchs should be studying other capsulated and hyaluronidase-producing strains like *Streptococcus pyogenes* (Hynes et al., 2000) and *Clostridium perfringens* (Canard et al., 1994).

Low temperature (4°C) of incubation was required to inhibit a rapid bacterial division. The increase of the number of transformants using pure hyaluronidase

was expected because of the direct effect of the enzyme with the substrat.
Natural transformation efficiencies are related to many factors, such as the nature and concentration of plasmids and the state of the plasmid DNA (super coiled, circular, linear, or multimeric form) (Norgard et al., 1978; Hanahan, 1983; Stewart and Carlson, 1986; Lorenz and Wackernagel, 1994).

Further studies, like it was done in 1996 from Baur et al. should be carried on to understand if there is correlation between Transformation frequency and

1. Ca^{2+} concentration
2. DNA concentration
3. Temperature shifts

Regarding the author's discussion on horizontal gene transfer in natural ecosystems, this also could be happening for other bacteria presenting hyaluronic acid-rich capsules in presence of those producing hyaluronidase. Future studies should be carried out to understand how natural genetic transformation affect's this type of bacteria.
On the basis the author's founding, it seems legitimate to presume that the natural development of genetic competence in *P. multocida X73* is biologically possible. In this case, the transformation process will be highly dependent on the phenotypical characteristics of each strain and on the type and characteristics of the transforming DNA.

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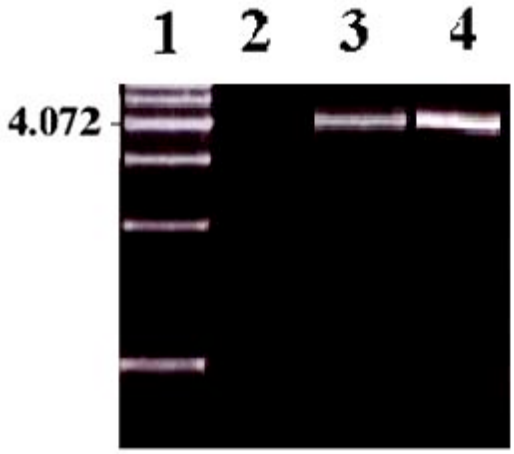

Figure 1. Agarose gel (0.7%) electrophoresis of the digested pPBA1100 with *Eco*RI (life technologies) isolated from *P. multocida* X73. Lane 1 = 1 kb ladder (life technologies); Lane 2 = No recombinants were produced in experiment using non-hyaluronidase-producing strains of *S. aureus*; Lane 3 = pPBA110 as positive control; Lane 4 = pPBA1100 isolated from recombinant *P. multocida* X73.

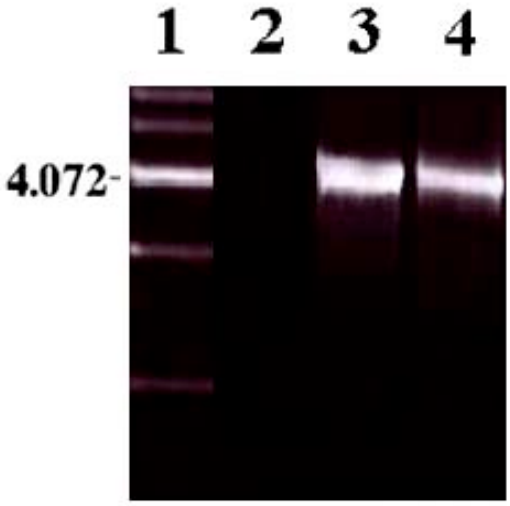

Figure 2. Agarose gel (0.7%) electrophoresis of the PCR-amplified pPBA110 (directly from single colonies grown on sheep blood agar plates from recombinants *P. multocida* X73) using the inverse primers pba-1 and pba-2. Lane 1 = 1 kb ladder (life technologies); Lane 2 = No recombinants were produced in experiment using non-hyaluronidase-producing strains of *S. aureus*; Lane 3 = pPBA110 as positive control. Lane 4 = pPBA1100 isolated from recombinant *P. multocida*X73.