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Expression of OmpP2A and OmpP2B is not required for pustule formation by *Haemophilus ducreyi* in human volunteers

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Abstract

Haemophilus ducreyi express two porin proteins, termed OmpP2A and OmpP2B. To test whether expression of OmpP2A and OmpP2B was necessary for virulence in humans, eight volunteers were experimentally infected with the parent (35000HP) in one arm and a double OmpP2A OmpP2B mutant (35000HP::P2AB) in the other arm. The pustule formation rates were 58.3% (95% CI, 33.2–83.5%) for the parent and 41.7% (95% CI, 19.3–64.0%) for the mutant ($P=0.25$). Biopsy of 35000HP and 35000HP::P2AB-infected sites yielded similar amounts of bacteria in quantitative culture. These results indicate that expression of OmpP2A and OmpP2B is not necessary to initiate disease or to progress to pustule formation in humans.

Keywords: Pustule formation; *Haemophilus ducreyi*; Porin proteins; Uirulence; Human challenge model

1. Introduction

Haemophilus ducreyi is a gram negative, unencapsulated bacterium that causes the genital ulcer disease chancroid. Chancroid is rare in the US but remains prevalent in some developing countries [1]. *H. ducreyi* is an important pathogen because it facilitates the acquisition and transmission of human immunodeficiency virus type 1 [1,2].

To study *H. ducreyi* pathogenesis in humans, we developed an experimental infection model in which 35000HP and its derivatives are delivered to the skin of the upper arms of healthy adult volunteers by puncture wounds made by an allergy-testing device [3]. Papules form within 24 h of inoculation and either evolve into pustules in 2–5 days or resolve spontaneously. There are significant effects of host, gender and dose on outcome in the model [4–6]. To test the role of putative virulence determinants in humans, mutant–parent comparison trials have been performed using the model [7]. In these trials, volunteers are inoculated with multiple doses of the parent on one arm and of the mutant on the other arm. Volunteers serve as their own controls for gender and host effects. A group of subjects is challenged with an EDD of the parent that causes a pustule formation rate of 70% (approximately 90 CFU) and with two-fold serial dilutions of the mutant that span the parent dose (180, 90 and 45 CFU). If similar pustule formation rates are observed for the parent and the mutant, we repeat the experiment. If the results are confirmed, we conclude that there is no major difference in the virulence of the mutant and parent and terminate the trial. If pustules do not develop at sites inoculated with the mutant, we increase the dose of the mutant in the next group(s) of subjects until the EDD of the mutant is at least 10-fold higher than that of the parent. These trials are usually accomplished with 6–9 subjects.

All mutants tested to date have been able to initiate papule formation. Mutants have fallen into three categories: virulent, defined as those that form pustules at doses similar to the

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parent; attenuated, those that do not form pustules even at doses approximately 10-fold that of the parent; and partially attenuated, those that only form pustules at doses two- to three-fold higher than the parent but not at doses equivalent to the parent. To date, of 16 mutants tested, those that lack an intact *flp* locus, expression of the hemoglobin receptor (HgbA), the peptidoglycan-associated lipoprotein (PAL), DsrA, an outer membrane protein (OMP) that is the major known determinant of serum resistance, LspA1 and LspA2, two large proteins necessary for evasion of phagocytosis, or NcaA, an OMP involved in collagen adherence, are attenuated [7–9] (unpublished). A mutant that lacked expression of DltA, which partially contributes to serum resistance, was partially attenuated [10].

H. ducreyi 35000HP (HP, human passaged) contains two homologous, contiguous genes, *ompP2A* and *ompP2B*, which encode protein products with estimated molecular masses of 46 and 43 kDa, respectively [11]. Reverse transcription PCR indicates that *ompP2A* and *ompP2B* are transcribed independently of one another. OmpP2A and OmpP2B have 27–33% homology to the P2 protein of *Haemophilus influenzae*, a classical trimeric porin. Purified OmpP2A and OmpP2B exhibit porin activity in black lipid bilayer assays and are the first porins described for *H. ducreyi* [11]. For other organisms, porins bear important immunogenic determinants, provide membrane stability, influence antibiotic susceptibility, and are targets of human bactericidal antibodies [12–15].

Western blot analysis of OMPs from different clinical isolates demonstrates that OmpP2A and OmpP2B are differentially expressed. A limited strain survey showed that of 10 *H. ducreyi* isolates, seven expressed OmpP2A, two expressed OmpP2B and one, 35000HP, expressed both proteins [11]. To examine whether either protein is required for virulence, we constructed a double mutant in the 35000HP background. We tested the mutant for the ability to cause disease in the human challenge model. To our knowledge, this is the first study evaluating the pathogenesis of a porin mutant in humans.

2. Results

2.1. Mutant characterization

Lipooligosaccharides (LOS) and OMPs were prepared from 35000HP and 35000HP::P2AB and subjected to SDS-PAGE analysis as described [16]. The LOS profiles demonstrated no differences between the mutant and the parent (data not shown). The OMP profile of 35000HP::P2AB was similar to that of 35000HP, except for the expected lack of expression of two bands with apparent molecular weights of approximately 40 and 45 kDa (Fig. 1). Western blot analysis using rabbit antisera to OmpP2A and OmpP2B [11] demonstrated a loss of reactivity to 35000HP::P2AB (Fig. 2). In addition, 35000HP and 35000HP::P2AB had identical generation times in broth cultures used to prepare the challenge inocula (data not shown).

For each iteration of the human challenge experiments, 35000HP and 35000HP::P2AB were tested for antibiotic susceptibilities to erythromycin, ciprofloxacin, and ceftriaxone using the E-test [17] and were shown to exhibit similar antibiotic sensitivity profiles (data not shown).

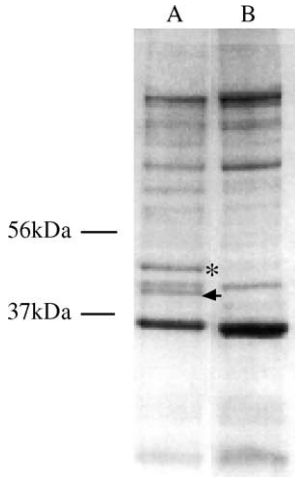

Fig. 1. SDS-10% PAGE and Coomassie blue staining of OMPs prepared from 35000HP (lane 1) and 35000HP::P2AB (lane 2). The asterisk represents OmpP2A and the arrow represents OmpP2B.

2.2. Human inoculation experiments

Eight healthy adults (four females, four males; age range 20–46; mean age ± the standard deviation, 28 ± 8.2) volunteered for the study. Two subjects (260 and 262) were challenged in the first iteration, three subjects (266, 267 and 268) were challenged in the second iteration and three subjects (261, 271 and 272) were challenged in the third iteration.

In the first iteration, we inoculated two subjects on one arm at three sites with 35000HP with an EDD of 82 CFU and on the other arm at three sites with 35000HP::P2AB with EDDs of 50, 101 and 203 CFU. Papules developed at six of six sites inoculated with the parent strain and five of six sites inoculated with the mutant strain (Table 1). Pustules developed at six of six sites inoculated with the parent strain (Table 1). A pustule

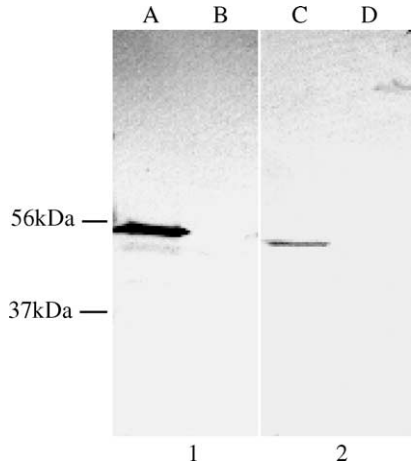

Fig. 2. Western blot probed with rabbit antisera to OmpP2A (panel 1) and OmpP2B (panel 2). Lanes A and C contain OMPs from 35000HP and lanes B and D contain OMPs from 35000HP::P2AB.