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Adaptive response to environmental changes in the fish pathogen *Moritella viscosa*

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Abstract

The marine psychrophilic bacterium *Moritella viscosa* is the causative agent of winter ulcer in farmed Atlantic salmon and cod. In this study, the growth requirements of the pathogen were established. The effects of changes in salinity and temperature on growth, surface features and proteomic regulation were also investigated. The genome of this bacterium has not yet been sequenced; therefore, comparative two-dimensional gel electrophoresis (2-DE) was used, coupled with high performance tandem mass spectrometry (MS/MS), to perform cross-species protein identification. Results from this study establish that *M. viscosa* is a true marine psychrophilic bacterium capable of surviving and proliferating in an oligotrophic and cold environment. Low temperature combined with 3–4% NaCl resulted in significantly higher cell yields and stability compared to high temperature and 1% NaCl. Nine cytoplasmic proteins were shown to be regulated by temperature and 12 by salinity. Several of the regulated proteins indicated a stressful situation at 15 °C compared to 4 °C, consistent with the growth characteristics observed. Furthermore, temperature and salinity were demonstrated to be important determinants of motility and viscosity of *M. viscosa*.

Keywords: Environmental stress response; Proteomics; Psychrophilic; *Moritella viscosa*

1. Introduction

The disease referred to as ''winter ulcer'', that affects farmed Atlantic salmon (*Salmo salar*) and farmed cod (*Gadus morhua*), is associated with low seawater temperatures (<7 °C) and is characterized by development of ulcers on the scaled parts of the body and around the eyes. Although other agents have been considered etiologically responsible for the disease, *Moritella viscosa* (formerly *Vibrio viscosus*) is now regarded as the main causative agent of winter ulcer [7,11,19,20,23]. Previous investigations of *M. viscosa* have focused mainly on phylogenetic classification and genotypic and phenotypic characterization of strains [2,21]. In contrast, information about growth requirements and mechanisms involved in its adaptation to different environmental conditions remains scarce [1].

Microorganisms generally experience limited availability of nutrients in their natural habitat, unlike in the laboratory environment, and have an immense capacity to survive and adapt to various environmental conditions. Temperature and salinity have been described as important environmental factors that affect bacterial growth, infection rates and adhesion processes of other *Vibrio* species [3,6,17].

The aims of this study were to determine the growth requirements of *M. viscosa* and to investigate the effects of changes in salinity and temperature on growth, surface features and the cytoplasmic proteome of the pathogen. A proteomic strategy using two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry (MS/MS) was employed to investigate differentially expressed proteins.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

Eight *M. viscosa* strains isolated from Atlantic salmon from different geographical locations and suffering from winter ulcer were included in this study: NVI 88/478^T, NVI 00179, NVI 4889, NVI 4950, NVI 4506, NVI 2759, LFI 5006 and LFI 5000. Prior to each experiment, strains from frozen stock cultures were grown on blood (5% bovine) agar plates (Oxoid base no. 2) supplemented with 2% NaCl. Pure colonies were cultured in marine broth (MB-2216, Difco) with agitation (200 rpm) for 24 h at 15 °C. Fresh overnight cultures were diluted 1:50 in various growth media and incubated further at 4 °C or 15 °C. Growth curves were established by measuring optical densities (ODs) at 600 nm with a UNICAM 8625 UV/vis spectrophotometer.

2.2. Amino acid, trace metal and nucleotide requirements

Requirements of amino acids, trace metals and nucleotides were studied using a defined salt broth (SB) (256.7 mM NaCl, 8.2 mM Na_2SO_4, 9.4 mM KCl, 49 mM $MgCl_2$, 6.8 mM $CaCl_2$, 3.6 μM $FeCl_3$, 18.7 mM NH_4Cl, 0.29 mM K_2HPO_4, 41.3 mM Trizma base, 0.5% glucose) [14] at 12–15 °C. The different supplements were added to individual flasks of SB. Stock solutions of trace metals and nucleotides were added to a final concentration of 1% [9], while casamino acids (Difco) were added to a final concentration of 0.07%. MB, SB and SB without glucose were used as controls.

Data were analyzed using Stata 9.0 (Stata Corp, College Station, TX). Descriptive analyses were undertaken using *x-y* plots between days and ODs for the different broths. Further analyses of data were undertaken using a linear regression approach where the relationship between OD and the explanatory variables (days, days squared, supplements) was modeled with strain as a cluster random effect. The final model was assessed for fit and residuals were tested using the normal quantile plot.

2.3. Temperature and salinity—effect on generation time, cell density and cell stability

Initial studies on the effect of temperature on growth were performed in SB at 4 °C and 15 °C. This was then elaborated to combine the effects of varying temperatures with varying salinities. These studies were performed in Luria broth (LB, 1–4% NaCl) at 4 °C and 15 °C to ensure sufficient amounts of nutrients.

Data were analyzed using *x–y* plots between days and OD for combinations of temperature and NaCl levels, and regression analysis was performed as described above.

2.4. Temperature and salinity—effect on motility and viscosity

2.4.1. Viscosity

Strains were cultivated on blood agar plates supplemented with different concentrations of NaCl at 4 °C and 15 °C. The viscosity was recorded after 4 days and graded from 0 to 4, where 4 represented highly viscous colonies which formed long strands or resisted separation when manipulated with a loop, and 0 represented no viscosity.

2.4.2. Motility

LB cultures (1–4% NaCl) at 4 °C and 15 °C were examined for motility by phase contrast microscopy on a daily basis for seven days and graded 0 to 4, where 4 represented numerous highly active cells and 0 represented no motility.

Further computational analyses were undertaken after graphical and tabular examination of data using the ordinal logistic procedure in Stata 9.0. The model was built by incorporating NaCl and temperature with strain as a cluster random variable. A backward selection procedure comparing log likelihoods was used to decide which predictor variable(s) were to be kept in the model. Prediction probabilities were established for each combination of factors.

2.5. Extraction of proteins and 2-DE separation

Strain LFI 5006 was cultivated to mid-exponential phase at 4 °C and 15 °C in LB with 1% and 3% NaCl, so as to mimic the osmotic concentrations in host bodies and marine waters, respectively. Cells were harvested by centrifugation and washed in low salt buffer (68 mM NaCl, 9 mM NaH_2PO_4, 3 mM KCl, 1.5 mM KH_2PO_4, pH 8). One ml lysis buffer (9 M urea, 4% CHAPS, 0.5% pharmalyte and 1% dithiothreitol (DTT)) was added per 200 μl cell pellet, and cells were disrupted by sonic oscillation on ice. One microliter of DNase I (Benzonase, Sigma, 25 U/μl) and 5 mM $MgCl_2$ were added prior to centrifugation (16,000 × *g*, 20 min) to remove particles. The protein concentrations were measured [4] and protein extracts (150 μg per sample) were diluted in buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10% glycerol, 0.5% pharmalyte, 0.2% DTT). Isoelectric focusing was performed in 13 cm pH 3–10 NL IPG strips (GE Healthcare, US) to 77,000 Vh. The strips were equilibrated by 15 min incubation in 50 mM Tris (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 2% DTT followed by 15 min in an identical buffer with 5% iodoacetamide as a substitute for DTT. Second-dimension separation was performed on 12% (w/v) SDS–polyacrylamide gels utilizing the PROTEAN II system (Bio-Rad Laboratories, CA, USA) in a Laemmli buffer system [15].

2.6. Visualization of protein spots and image analysis

Proteins were stained with Coomassie blue (Invitrogen, CA, US) and images captured by the Fluor-S™ MultiImager (Bio-Rad). Images were processed using PDQuest software, version 6.0 (Bio-Rad). Groups were created consisting of four gels from each environmental condition. The different groups were compared and differences were tested statistically using a standard setup of Student's *t*-test with a significance level of 0.05.