Microbial Pathogenesis 46 (2009) 21–27

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Flavobacterium columnare colony types: Connection to adhesion and virulence?

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ARTICLE INFO

Article history:
Received 21 May 2008
Received in revised form
3 September 2008
Accepted 4 October 2008
Available online 17 October 2008

Keywords:
Flavobacterium columnare
Colony morphology
Virulence
Adhesion
Phase variation

ABSTRACT

Four different colony morphologies were produced by *Flavobacterium columnare* strains on Shieh agar plate cultures: rhizoid and flat (type 1), non-rhizoid and hard (type 2), round and soft (type 3), and irregularly shaped and soft (type 4). Colonies produced on AO agar differed from these to some extent. The colony types formed on Shieh agar were studied according to molecular characteristics [Amplified Fragment Length Polymorphism (AFLP), Automated Ribosomal Intergenic Spacer Analysis (ARISA), and whole cell protein SDS-PAGE profiles], virulence on rainbow trout fingerlings, and adhesion on polystyrene and fish gills. There were no molecular differences between colony types within one strain. Type 2 was the most adherent on polystyrene, but type 1 was the most virulent. Adhesion of *F. columnare* strains used in this study was not connected to virulence. From fish infected with colony type 1, three colony types (types 1, 2 and 4) were isolated. Contrary to previous studies, our results suggest that strong adhesion capacity may not be the main virulence factor of *F. columnare*. Colony morphology change might be caused by phase variation, and different colony types isolated from infected fish may indicate different roles of the colony morphologies in the infection process of columnaris disease.

1. Introduction

Flavobacterium columnare, the causative agent of columnaris disease, is a freshwater bacterium that can be isolated from natural waters [1,2]. Nowadays, the bacterium is considered as one of the most harmful bacterial fish pathogen at freshwater fish farms worldwide: columnaris outbreaks cause remarkable financial and material losses yearly for the fish farming industry (see Ref. [3]). Development of improved cultivation methods (*e.g.* Refs. [4,5]) has enabled a routine isolation of *F. columnare*, facilitating the diagnostics and studying of this pathogen in laboratory conditions.

Despite its pathogenicity, virulence mechanisms of *F. columnare* are largely unknown. It is known, that different genetic groups [6–8] express different degrees of virulence. It has also been shown that the activity of connective tissue degrading enzyme, chondroitin AC lyase [7], and capacity to adhere on gill tissue [9] are related to virulence of *F. columnare*. Growth characteristics in different culture conditions [10], production of extracellular proteases [11,12] and outer membrane protease genes [13] have been studied, but clear correlation to virulence has not been observed. On the other hand, differences in lipopolysaccharide (LPS) and protein profiles between virulent and avirulent *F. columnare* strains have been detected [14]. In some pathogenic bacteria, cell surface components, such as LPS and capsular material, function as virulence factors (*e.g.* Refs. [15–17]).

Change in cell surface components often leads to change in colony morphology of a bacterium (see *e.g.* Refs. [16–19]). In some human and animal (also fish) pathogens, such as tubercle bacilli [20], *Vibrio vulnificus* [21], and *Mycobacterium avium* [22], different colony morphologies produced by one strain can exhibit difference in virulence. Previously, difference in colony morphology has been detected between *F. columnare* strains [23]. Also non-rhizoid as well as soft and non-adherent colonies have been noticed to appear among rhizoid colonies after subcultivation [24,25]. It has been shown, that spreading or rhizoid colony formation is an indication of gliding motility of *Flavobacterium johnsoniae* (previously *Cytophaga johnsonae*) [26] and *Flavobacterium psychrophilum* (previously *Cytophaga psychrophila*) [27]. Therefore, it is possible, that the loss of gliding motility appears as non-rhizoid colony morphology also in *F. columnare*. Different colony morphologies have also been found in *Flavobacterium succinicans* (previously *Cytophaga succinicans*), a relative to *F. columnare* [28]. However, the pathogenesis of colony morphology variants has not been studied in flavobacteria.

We found that four different colony morphologies are formed among *F. columnare* strains on Shieh agar plates in laboratory cultivations, and one strain can form one or two morphology variants. Because of severity of repeated infections at fish farms and lack of knowledge of virulence mechanisms in *F. columnare*, we consider it important to study further these colony types. This is necessary both for developing exact diagnostic tools for columnaris infections and also to find out whether there is a connection

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doi:10.1016/j.micpath.2008.10.001

between virulence and colony morphology. This is why molecular characteristics and adhesion properties as well as fish mortality caused by different colony morphologies were studied.

2. Results

Four different colony types (Fig. 1) were formed on Shieh agar plates in laboratory conditions by the eight *F. columnare* strains tested (Table 1). Colony type 1 was rhizoid and flat with yellow centre. Colony type 2 was hard, more orange in color, non-rhizoid or only slightly rhizoid, and had irregular edges and convex growth form. Colony type 3 had round edges, and smooth, yellowish appearance. Type 4 colonies were white or light yellow, smooth and spreading on the agar with irregular shape. Originally, *F. columnare* strains formed colony types 1–4 (Table 1), each strain producing only one colony type. In further plate cultivations other colony types started to form among original types in a following manner: type 1 → type 2, type 2 → type 4, and type 3 → type 4, meaning that among type 1 colonies, type 2 colonies started to appear, etc. These other colony types all formed in the cultures of same age and, once formed, remained the same from generation to generation in laboratory cultivations. There was no colony type change in the opposite direction meaning, that no type 3, 2 or 1 colonies were formed among type 4 colonies, no type 2 or 1 colonies among type 3 colonies, and no type 1 colonies among type 2 colonies. Colony type did not change in broth cultivations. In older cultures (more than 3 days after plate cultivation), however, on the edges of some types 2 and 3 colonies growth resembling type 4 started to appear. There were no contaminations of other bacteria or between *F. columnare* strains in the cultures. Colony type 4 existed only in the genetic groups with low or intermediate virulence, whereas colony type 3 existed only in high virulence strain. On AO agar, formation of colony morphologies differed from that on Shieh agar to some extent: The growth form of bacteria was more spreading on AO agar than on Shieh agar. Moreover, the older cultures on AO agar started to get transparent appearance, which did not occur on Shieh agar. The growth form of all bacteria of all genetic groups resembled colony type 4 formed on Shieh agar, but had more spreading colonies with slightly rhizoid edges. The exceptions were genetic groups B and F, which formed types 3 and 4 (formed on Shieh agar) resembling colonies also on AO agar, respectively. Among rhizoid type 4 colonies, also types 1 and 2 (formed on Shieh agar) resembling colonies were formed by bacteria of genetic groups D, E and G. None of the colony types 1–4 formed on AO agar had exactly the same appearance as the corresponding colony types 1–4 formed on Shieh agar, but could be categorized into these groups. However, because of the spreading growth form of bacteria on AO agar, the different colony types grew rather stuck on each other than as separate colonies. On AO agar, the colony types 1, 2 and 4 formed originally on Shieh agar grew as rhizoid type 4 colonies. Types 1 and 2 (formed on Shieh agar) resembling colonies were also detected among D2 (colony type 2 formed by strain D), E1, E2 and G2.

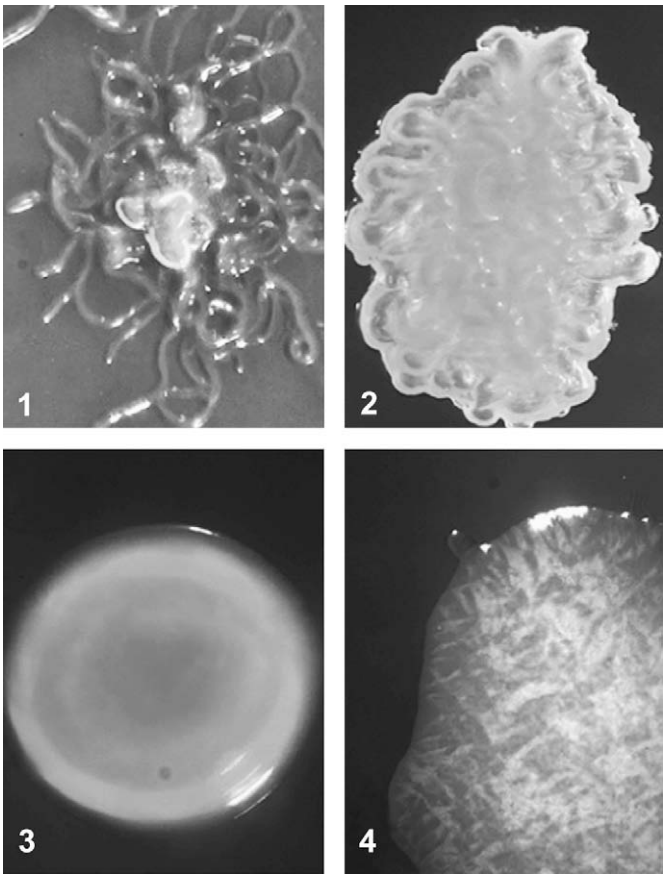

Fig. 1. Representatives of different colony morphologies (1 = colony type 1, 2 = type 2, 3 = type 3, 4 = type 4) formed by *Flavobacterium columnare* (see also Table 1) on Shieh agar plate cultivation in laboratory. Type 1 is rhizoid and flat with yellow centre. Type 2 is hard, more orange in color, non-rhizoid or only slightly rhizoid, and has irregular edges and convex growth form. Type 3 has round edges, and smooth, yellowish appearance. Type 4 colonies are white or light yellow, smooth and spreading on the agar with irregular shape.

There were no differences in ARISA [10], AFLP or whole cell protein SDS-PAGE (Fig. 2) profiles between different colony types formed by one *F. columnare* strain. However, there were differences in adhesion capacities on polystyrene (Kruskal–Wallis test, overall comparison between strains A–H forming colony types 1–4: $\chi^2 = 66{,}947$, df = 12, $P < 0.001$) (Fig. 3a): D2 and D4, E1 and E2, G2 and G4, as well as H2 and H4 differed significantly from each other. Similarly, on polystyrene, there was a significant difference between adhesion capacities of colony types (one-way ANOVA: $F = 36.679$, df = 4, $P < 0.001$) (Fig. 3b); colony type 2 was more adherent than other colony types, but also the adhesion of colony type 1 differed significantly from the other colony types. The growth rate of bacteria did not affect the adhesion capacities. On agar, colony type 2 was the most adherent followed by types 1, 3 and 4. This result was achieved by the experience when handling the colonies with inoculation loop. Adhesion capacities on gill tissue differed significantly between colony types among the strains (one-way ANOVA: $F = 3{,}306$, df = 6, $P = 0.031$) (Fig. 3c). G4 was the most adherent and differed significantly from all the other colony types tested, except from E2. A significant difference between adhesion capacities of E2 and H4 was also detected.

In challenge experiment, there was a significant difference between mortalities caused by the colony types (Kaplan–Meier

Table 1
Colony morphology types formed on Shieh agar in laboratory conditions by genetically grouped *Flavobacterium columnare* strains (see Refs. [10] and [7] for more details on the strains).

Genetic group	Virulence of the strain	Original colony type	Colony type formed in subcultivations
A	Low	1	
B	High	3	
C	High	1	
D	Low	2	4
E	High	1[a]	2[a]
F	Low	4	
G	Intermediate	2[a]	4[a]
H	Low	2[a]	4[a]

[a] Colony type was used in the virulence experiment using rainbow trout (*Oncorhynchus mykiss*, Walbaum) fingerlings.