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Antigenic characterization of *Enterococcus* strains pathogenic for turbot and their relationship with other Gram-positive bacteria

Alicia E. Toranzo, Juan M. Cutrín, Soledad Núñez, Jesús L. Romalde, Juan L. Barja

Departamento de Microbiología y Parasitología, Facultad de Biología, Universidad de Santiago de Compostela, E-15706 Santiago de Compostela, Spain

ABSTRACT: We describe here the serological and antigenic characterization of a group of highly virulent *Enterococcus* strains that, since 1993, have caused heavy economic losses in turbot *Scophthalmus maximus* reared in northern and northwestern Spain. Reference Gram-positive strains and the corresponding polyclonal antisera raised against some species of lactic acid bacteria as well as *Corynebacterium aquaticum* and *Renibacterium salmoninarum* were included. Agglutination assays demonstrated that regardless of the farm and year of isolation, all strains from turbot were serologically homogeneous. However, a number of cross-reactions occurred among the various groups of bacteria (mainly among *Enterococcus*, *Carnobacterium* and *Lactobacillus* species). Analysis of the surface proteins and western blot assays supported the antigenic homogeneity of the *Enterococcus* strains isolated from turbot because they all yielded an essentially identical protein pattern (major common bands were 40, 45, and 50 kDa) and reacted similarly with antisera raised against representative strains of this group (there were at least 7 common bands of 20, 25, 30, 40, 45, 57 and 80 kDa). This serological homogeneity suggests that the development of vaccines to control enterococcosis in turbot should be feasible. Irrespective of the source of the polyclonal antisera employed, common immunoreactive proteins were present in all the Gram-positive bacilli and cocci tested. This, together with the cross-agglutination reactions noted among the various bacterial taxa tested, suggests that serological identification of the turbot *Enterococcus* using polyclonal antisera could be problematical.

KEY WORDS: *Enterococcus* · Turbot · Serology · Antigenic proteins · Gram-positive bacteria

INTRODUCTION

Although streptococcosis and enterococcosis are common infections among freshwater and marine fish species all over the world (see reviews of Austin & Austin 1993, Kitao 1993, Kusuda & Salati 1993), it is in Japan where these diseases cause major losses, especially in yellow tail *Seriola quinqueradiata*. In Spain, the isolation of such bacteria from dying, farmed turbot *Scophthalmus maximus* was very uncommon until 1992 (Toranzo et al. 1990, 1993a, 1994a); however, this situation now appears to be changing.

We recently described the first occurrence of an enterococcal septicaemia in turbot cultured in northwestern Spain (Toranzo et al. 1994b). Although the disease was first diagnosed in 1992 in only 1 farm, since 1993 it has become one of the most important bacterial problems in a great number of ongrowing turbot facilities, causing heavy economic losses. Chemotherapy has not proved effective at preventing mortalities and, therefore, vaccination seems to offer the best prospects for controlling the disease.

The most noticeable clinical signs in affected fish (from 50–100 g to 2–3 kg) are pronounced uni- or bilateral exophthalmia with an accumulation of mucupurulent exudate in the eyeball. This accumulation of purulent fluid also frequently occurs at the base of the pectoral fins (Toranzo et al. 1994b). The histopathology of this enterococcal infection in turbot has already been described (Nieto et al. 1995).

Biochemical characterization of the turbot isolates (Toranzo et al. 1994b) revealed that all of them, regardless of the source, exhibited the same phenotypic pattern and they were classified as an *Enterococcus* species, closely related with the fish pathogen *Enterococcus seriolicida* (Kusuda et al. 1991) and *Lactococcus*

Table 1 Slide agglutination reactions and cross-agglutination titers among the Gram-positive bacteria used in this study. Titers (in parentheses) are expressed as the reciprocal of the highest dilution of antiserum that gave a positive reaction after overnight incubation at 30°C. –: titers less than 2

Bacteria	Origin	Source of rabbit antiserum							Streptococcal grouping kit (Oxoid)
		Enterococcus sp.		*Enterococcus seriolicida*	*Carnobacterium piscicola*		*Corynebacterium aquaticum*	*Renibacterium salmoninarum*	
		RA-99.1	AZ-12.1	ATCC 49156	ATCC 35586	HB-426	RB-968 BA	ATCC 33209	
Enterococcus sp.									
RA-83.1	Turbot (farm A, 1992), Spain	+ (256)	+ (256)	–	–	–	–	–	–
RA-99.1	Turbot (farm A, 1993), Spain	+ (512)	+ (256)	–	–	–	–	–	–
AZ-12.1	Turbot (farm B, 1993), Spain	+ (512)	+ (512)	–	–	–	–	–	–
RM-207.1	Turbot (farm C, 1993), Spain	+ (256)	+ (256)	–	–	–	–	–	–
RA-100.1	Turbot (farm A, 1994), Spain	+ (512)	+ (256)	–	–	–	–	–	–
AZ-71.1	Turbot (farm B, 1994), Spain	+ (256)	+ (512)	–	–	–	–	–	–
RM-212.1	Turbot (farm C, 1994), Spain	+ (512)	+ (512)	–	–	–	–	–	–
RIM-17.1	Turbot (farm D, 1994), Spain	+ (512)	+ (512)	–	–	–	–	–	–
RP-85.1	Turbot (farm E, 1994), Spain	+ (512)	+ (256)	–	–	–	–	–	–
Enterococcus seriolicida									
ATCC 49156	Yellow tail, Japan	–	–	+ (1024)	+ (512)	+ (32)	+ (512)	–	–
SS-91014	Yellow tail, Japan	–	–	(+) (16)	+ (128)	–	–	–	–
Enterococcus sp.									
TW-1	Rainbow trout, Spain	–	–	–	–	–	–	–	–
Lactococcus garvieae									
NCDO 2155	Bovine, UK	–	–	+ (512)	+ (128)	–	+ (128)	–	–
Enterococcus faecalis									
ATCC19433	Human, France	–	–	–	+ (512)	–	–	–	D
Streptococcus sp.									
117	Striped bass, USA	–	–	–	–	–	–	–	B
Streptococcus pyogenes									
X66	Human, Spain	–	–	–	–	–	–	–	A
Carnobacterium piscicola									
ATCC 35586	Cutthroat trout, USA	+ (64)	+ (128)	+ (512)	+ (1024)	–	–	–	D
HB-426	Striped bass, USA	–	–	+ (512)	+ (64)	+ (2048)	–	–	D
PT-31	Rainbow trout, Spain	–	–	+ (512)	–	–	–	–	D
Carnobacterium sp.									
RPM-574.1	Fish food, Spain	+ (128)	+ (128)	+ (128)	–	+ (512)	–	–	D
Lactobacillus casei									
ATCC 393	Cheese, USA	+ (128)	+ (64)	+ (128)	+ (512)	+ (512)	+ (512)	–	G
Corynebacterium aquaticum									
RB-968 BA	Striped bass, USA	–	–	–	–	–	+ (2048)	–	–
ATCC 14665	Distilled water, USA	–	–	–	–	–	+ (256)	–	–
Renibacterium salmoninarum									
ATCC 33209	Pacific salmon, USA	–	–	–	–	–	–	+ (4096)	–
ATCC 33739	Brook trout, USA	–	–	–	–	–	–	+ (4096)	–
MT-251	Atlantic salmon, UK	–	–	–	–	–	–	+ (2048)	–
466	Pacific salmon, Canada	–	–	–	–	–	–	+ (2048)	–
RS-92	Pacific salmon, Spain	–	–	–	–	–	–	+ (4096)	–

garvieae, an organism thus far isolated from bovine mastitis and human clinical material (Garvie et al. 1981, Elliot et al. 1991).

The aim of this work was 2-fold. First, we wished to determine whether all of the *Enterococcus* strains isolated from turbot in different years and farms were antigenically homogeneous. Second, we wished to determine whether the *Enterococcus* strains were serologically distinct from other important Gram-positive fish pathogens. It was visualized that the results would be relevant to such problems as the detection and identification of the turbot pathogen and to the development of vaccines for preventing enterococcosis in turbot.

MATERIALS AND METHODS

Bacterial strains and culture conditions. We selected a group of representative strains of *Enterococcus* sp. strains isolated in northern and northwestern Spain from moribund turbot in different farms and years (see Table 1). As previously reported (Toranzo et al. 1994b), these strains were identified using standard morphological, physiological and biochemical plate and tube tests (Colman et al. 1992, Devriese et al. 1993, Holt et al. 1994, Smibert & Krieg 1994), and by using the commercially available API-20 Strep and API-50 CHL systems (bioMerieux, Madrid, Spain).

To determine the serological and antigenic relationships with other Gram-positive bacterial groups, reference strains belonging to species of the genera *Enterococcus*, *Streptococcus*, *Lactococcus*, *Carnobacterium*, *Lactobacillus*, *Corynebacterium* and *Renibacterium* were studied. The origin of the bacteria is listed in Table 1.

The bacteria were routinely cultured on brain heart infusion agar (BHIA, Difco Laboratories, Detroit, MI, USA) at 25°C for 48 to 72 h. In the case of *Renibacterium salmoninarum*, strains were grown on Mueller-Hinton agar (Difco) supplemented with 0.1% L-cysteine hydrochloride (MHA-C) at 15°C for about 10 d. All bacterial isolates were stored frozen in their respective broth media containing 15% (v/v) glycerol until tested.

Serological characterization. To investigate the serological relatedness among the *Enterococcus* strains from turbot and to examine their relationship with other Gram-positive bacteria, slide agglutination tests were conducted as previously described (Toranzo et al. 1987) using polyclonal rabbit antisera raised against the following representative strains of each group (Table 1): *Enterococcus* sp. RA-99.1 and AZ-12.1 (turbot isolates); *E. seriolicida* ATCC 49156 (YT-3^T); *Carnobacterium piscicola* ATCC 35586 (B-270^T) and HB-426; *Corynebacterium aquaticum* RB-968 BA; and *Renibacterium salmoninarum* ATCC 33209 (Lea-74^T). In addition, cross-quantitative agglutination assays were performed in microtiter plates using serial 2-fold dilutions of 25 µl aliquots of each antiserum. The agglutination titer was defined as the reciprocal of the highest dilution of the antiserum that gave a positive reaction after overnight incubation with the antigen at 30°C. In all assays, the test bacteria suspended in saline were used as controls.

A commercially available streptococcal serotyping kit (Oxoid, DR 585A, Basingstoke, England), capable of identifying Lancefield serotypes A to G, was used on all of the bacteria in Table 1.

Analysis of surface proteins and western blotting. Cell surface proteins from all of the Gram-positive bacilli and cocci were prepared as previously described (Bandín et al. 1993, Toranzo et al. 1993b). Samples were electrophoresed in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) at constant current (Laemmli 1970). The proteins were stained with Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, MO, USA), and the molecular masses (MM) were determined by comparison with a mixture of MM markers (Bio-Rad).

After electrophoresis, proteins were electroblotted from the gels onto nitrocellulose (NC) membranes (Bio-Rad) using the method of Towbin et al. (1979). The NC membranes were then separately reacted with the different polyclonal antisera mentioned above. Reacting protein bands were visualized as previously described (Bandín et al. 1993, Toranzo et al. 1993b).

RESULTS AND DISCUSSION

The agglutination assays (Table 1) indicated that all of the *Enterococcus* strains from turbot were serologically homogeneous. All of them displayed titers ranging from 256 to 512 with the antisera raised against representative isolates of this group (RA-99.1 and AZ-12.1) and none of them reacted with polyclonal antisera raised against the other Gram-positive bacteria (Table 1). However, polyclonal antisera raised against the turbot *Entercoccus* sp. cross-reacted with some strains of another fish pathogen (*Carnobacterium piscicola*), and a number of cross-reactions also occurred among the other Gram-positive bacteria tested, mainly among the *Enterococcus*, *Carnobacterium* and *Lactobacillus* species. Interestingly, the commercial streptococcal grouping kit did not yield reactions with the turbot enterococcus but it showed that all the *Carnobacterium* strains and *Enterococcus faecalis* ATCC 19433 behaved like group D streptococci. It was clear, therefore, that this kit, which was devised for

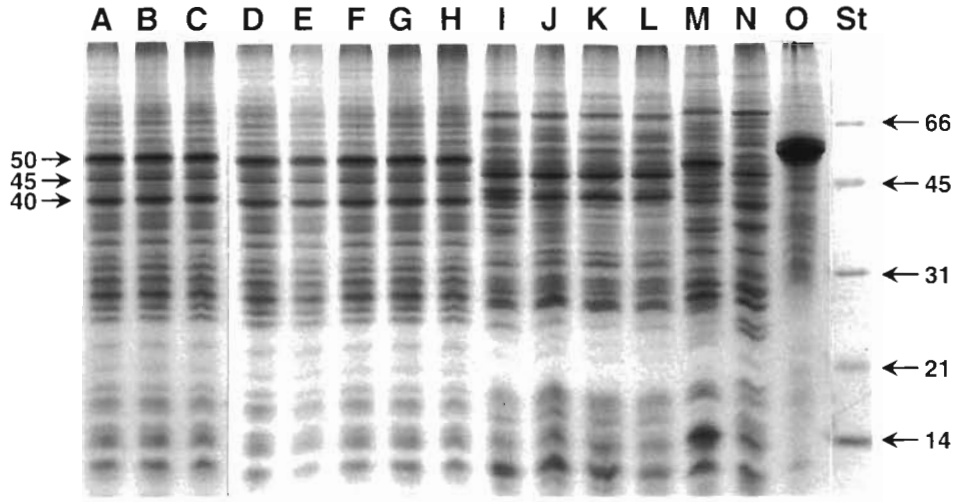

Fig. 1. SDS-PAGE of cell-surface proteins of the *Enterococcus* sp. strains from turbot *Scophthalmus maximus* and other selected Gram-positive bacteria. Lanes: (A to H) turbot isolates RA-83.1, RA-99.1, AZ-12.1, RM-207.1, RA-100.1, AZ-71.1, RM-212.1 and RIM-17.1; (I) *E. seriolicida* ATCC 49156; (J) *E. seriolicida* SS-91014; (K) *Enterococcus* sp. TW-1; (L) *Lactococcus garvieae* NCDO 2155; (M) *E. faecalis* ATCC 19433; (N) *Carnobacterium piscicola* HB-426; (O) *Renibacterium salmoninarum* ATCC 33209. St: standard of molecular mass markers in kDa. Arrows indicate some of the major proteins shared by the *Enterococcus* sp. from turbot

serotyping clinical isolates from mammals, was not suitable for serotyping the *Streptococcus-Enterococcus* strains from fish.

Analysis of the cell surface proteins revealed 2 main patterns. One of them was exhibited by all of the *Enterococcus* strains from turbot which presented an essentially identical profile displaying major common bands of 40, 45 and 50 kDa (Fig. 1, lanes A to H). The second pattern yielded by *Enterococcus* TW-1, *E. seriolicida* ATCC 49156 and SS-91014 strains, and *Lactococcus garvieae* NCDO 2155 contained major common proteins of 40, 47 and 80 kDa (Fig. 1, lanes I to L). The latter pattern of surface proteins supported the phylogenetic relationship recently described between the species *E. seriolicida* and *L. garvieae* (Doménech et al. 1993). The remaining Gram-positive bacteria tested displayed protein profiles unique to each strain (Fig. 1, lanes M to O).

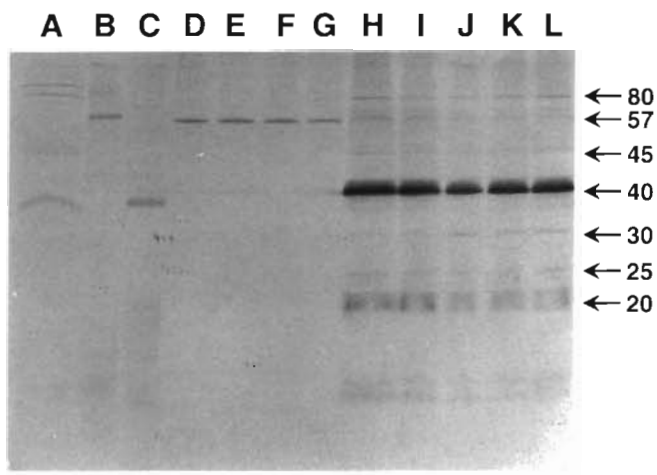

Fig. 2. Western blot of surface proteins of the Gram-positive bacteria using the rabbit polyclonal antiserum raised against the *Enterococcus* sp. RA-99.1 isolated from turbot *Scophthalmus maximus*. Lanes: (A) *Renibacterium salmoninarum* ATCC 33209; (B) *Carnobacterium piscicola* HB-426; (C) *E. faecalis* ATCC 19433; (D) *Lactococcus garvieae* NCDO 2155; (E) *Enterococcus* sp. TW-1; (F) *E. seriolicida* SS-91014; (G) *E. seriolicida* ATCC 49156; (H to L) *Enterococcus* sp. from turbot RA-99.1, AZ-12.1, RA-100.1, AZ-71.1, RM-212.1. Arrowheads show some of the immunoreactive proteins common to all the turbot isolates; molecular mass values in kDa

The western blot assays corroborated the results of the surface protein analysis and agglutination tests in that all of the *Enterococcus* strains from turbot reacted similarly with the antisera raised against representatives of this group: they shared at least 7 antigenic bands of about 20, 25, 30, 40, 45, 57 and 80 kDa (Fig. 2, lanes H to L). However, regardless of the polyclonal antiserum used, common immunoreactive proteins were present in many of the Gram-positive bacteria tested, even in strains that did not exhibit any cross-reaction in the agglutination assays (data not shown). The lack of correlation observed in this study between the cross-agglutination assays and the western-blot reactions can be explained, as previously indicated (Toranzo et al. 1993b), if it is assumed that the majority of the common antigenic proteins occur in low amounts or are not completely exposed to the antibodies; these antigens can therefore only be detected using a very sensitive procedure such as immunoblotting.

We conclude that the great antigenic homogeneity evident among our *Enterococcus* isolates from turbot should facilitate the development of vaccines for protecting turbot against enterococcosis. However, the cross-reactions found among the Gram-positive bacteria will make serological diagnosis of enterococcosis difficult, especially using polyclonal antisera. On the other hand, in agreement with other reports (Baya et al. 1991, Elliot et al. 1991, Carson et al. 1993,

Doménech et al. 1993, Toranzo et al. 1993b, Merquinor et al. 1994) we consider that analysis of electrophoretic cell protein profiles is a useful tool for establishing relatedness among lactic acid bacteria. Overall, however, the results of this and earlier studies on the turbot *Enterococcus* indicate that definitive criteria for identifying this pathogen cannot be based only on phenetic, serologic and antigenic data. Genetic data, based on DNA hybridization and analysis of 16 rRNA sequences, are needed to identify the bacterium and to better define the relationships among the pathogenic Gram-positive bacilli and cocci.

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LITERATURE CITED

Austin B, Austin DA (1993) Gram positive bacteria: the lactic acid bacteria. In: Austin B, Austin DA (eds) Fish pathogens. Disease in farmed and wild fish. Ellis Horwood, Chichester, p 23–42

Bandín I, Santos Y, Barja JL, Toranzo AE (1993) Presence of a common antigen among *Renibacterium salmoninarum, Corynebacterium aquaticum* and *Carnobacterium piscicola* using the western blot technique. J aquat Anim Hlth 5:172–176

Baya AM, Toranzo AE, Lupiani B, Li T, Roberson BS, Hetrick FM (1991) Biochemical and serological characterization of *Carnobacterium* spp., isolated from farmed and natural populations of striped bass and catfish. Appl environ Microbiol 57:3114–3120

Carson J, Gukovs N, Austin B (1993) Characteristics of an *Enterococcus*-like bacterium from Australia and South Africa, pathogenic for rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 16:381–388

Colman G, Efstratiou A, Morrison D (1992) *Streptococcus* and related organisms. In: Board RG, Jones D, Skinner FA (eds) Identification methods in applied and environmental microbiology. Blackwell, Oxford, p 221–249

Devriese LA, Pot B, Collins MD (1993) Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. J appl Bacteriol 75:399–408

Doménech A, Prieta J, Fernández-Garayzábal JF, Collins MD, Jones D, Domínguez L (1993) Phenotypic and phylogenetic evidence for a close relationship between *Lactococcus garvieae* and *Enterococcus seriolicida*. Microbiol Soc Espan Microbiol 9:63–68

Elliot JA, Collins MD, Piggott NE, Facklam RR (1991) Differentiation of *Lactococcus lactis* and *Lactococcus garvieae* from humans by comparison of whole cell protein profile patterns. J clin Microbiol 29:2731–2734

Garvie EI, Farrow JAE, Phillips BA (1981) A taxonomic study of some strains of streptococci which grow at 10°C but not at 45°C including *Streptococcus lactis* and *Streptococcus cremoris*. Zent Bakt Mikrobiol Hyg, 1 Abt Orig C 2: 151–165

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th edn. Williams & Wilkins Co., Baltimore, p 527–570

Kitao T (1993) Streptococcal infections. In: Inglis V, Roberts RJ, Bromage NR (eds) Bacterial diseases of fish. Blackwell, Oxford, p 196–210

Kusuda R, Kawai K, Salati F, Banner CR, Fryer JL (1991) *Enterococcus seriolicida* sp. nov., a fish pathogen. Int J syst Bacteriol 41:406–409

Kusuda R, Salati F (1993) Major bacterial diseases affecting mariculture in Japan. In: Faisal M, Hetrick FM (eds) Annual review of fish diseases, Vol 3. Pergamon Press, New York, p 9–85

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Merquinor VLC, Peralta JP, Facklam RR, Teixeira LM (1994) Analysis of electrophoretic whole-cell protein profiles as a tool for characterization of *Enterococcus* species. Curr Microbiol 28:149–153

Nieto JM, Devesa S, Quiroga I, Toranzo AE (1995) Histopathology of *Enterococcus* sp. infection in farmed turbot (*Scophthalmus maximus*, L.). J Fish Dis 18:21–30

Smibert RM, Krieg NR (1994) Phenotypic characterization. In: Gerhardt P, Murray RGE, Wood WA, Krieg NE (eds) Manual of methods for general and molecular bacteriology. American Society of Microbiology, Washington, DC, p 607–682

Toranzo AE, Baya AM, Roberson BS, Barja JL, Grimes DJ, Hetrick FM (1987) Specificity of the slide agglutination test for detecting bacterial fish pathogens. Aquaculture 61:81–97

Toranzo AE, Devesa S, Barja JL (1994a) An overview of the main infectious problems in cultured turbot: present status and future necessities. In: Lavens P, Remmerswaal R (eds) Turbot culture: problems and prospects. Proc. Turbot Workshop, World Aquaculture Congress, Torremolinos, Spain, May 1993. Eur Aquac Soc Spec Publ 22, Gent, p 106–126

Toranzo AE, Devesa S, Heinen P, Riaza A, Núñez S, Barja JL (1994b) Streptococcosis in cultured turbot caused by an *Enterococcus*-like bacterium. Bull Eur Ass Fish Pathol 14:19–23

Toranzo AE, Novoa B, Núñez S, Romalde JL, Devesa S, Mariño E, Silva R, Martínez E, Figueras A, Barja JL (1993a) Microflora associated to healthy and diseased turbot (*Scophthalmus maximus*) from three farms in northwest Spain. Aquaculture 114:189–202

Toranzo AE, Romalde JL, Núñez S, Figueras A, Barja JL (1993b) An epizootic in farmed, market-size rainbow trout in Spain caused by a strain of *Carnobacterium piscicola* of unusual virulence. Dis aquat Org 17:87–99

Toranzo AE, Santos Y, Bandín I, Romalde JL, Ledo A, Fouz B, Barja JL (1990) Five-year survey of bacterial fish infections in continental and marine aquaculture in northwest Spain. World Aquacult 21:91–94

Towbin H, Stachelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc natl Acad Sci USA 76:4350–4354

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