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Scanning Electron Microscopy of "Saddleback" Lesions Associated with Experimental Infections of *Flavobacterium columnare* in Channel Catfish, *Ictalurus punctatus* (Siluriformes: Ictaluridae), and Zebrafish, *Danio rerio* (Cypriniformes: Cyprinidae)

Stephen A. Bullard[1] and Andrew McElwain

Aquatic Parasitology Laboratory, Department of Fisheries and Allied Aquacultures, Auburn University, 324 Upchurch Hall, Auburn, Alabama 36849, USA

Covadonga R. Arias

Aquatic Microbiology Laboratory, Department of Fisheries and Allied Aquacultures, Auburn University, 203 Swingle Hall, Auburn, Alabama 36849, USA

Abstract

Laboratory-reared, specific pathogen-free fingerling channel catfish, *Ictalurus punctatus*, and adult zebrafish, *Danio rerio*, were each separately exposed by immersion challenge to the etiological agent of "columnaris disease," *Flavobacterium columnare* (Japanese Collection of Microorganisms 21327 strain). At 24-h post-immersion, fish exhibiting a "saddleback" lesion were fixed whole in 10% neutral buffered formalin. Skin samples approximately 5 mm^2 were excised from both the margin and center of each saddleback lesion as well as from corresponding sites in control, non-challenged, fish before being prepared routinely for scanning electron microscopy (SEM). Skin samples from control channel catfish and zebrafish had uniform, contiguous epidermal cells with continuous or closely apposed cell margins and well-defined microridges. Channel catfish skin lesion samples had margins typified by epidermal sloughing and lesion centers that exhibited a multitude of rod-shaped bacterial cells, approximately 3–10 μm long × 0.3–0.5 μm wide, intermingled with cellular debris across a surface characterized by denuded, strongly ridged, or folded dermal connective tissue. Zebrafish skin lesion samples had a multitude of rod-shaped bacterial cells and exhibited comparable ultrastructural changes but some lacked scales. These findings are the first published SEM observations of columnaris disease and saddleback lesions in channel catfish and zebrafish and thereby advance our understanding of the ultrastructural characteristics of acute-stage saddleback lesions and columnaris disease pathogenesis.

Flavobacterium columnare, the etiological agent of "columnaris disease," is a Gram-negative bacterium of the Cytophaga–Flavobacterium–Bacteroides group (Bernardet 1989; Bernardet and Grimont 1989; Bernardet et al. 1996). The bacteria is a well-recognized disease agent in aquaculture-reared and ornamental freshwater fishes from tropical and temperate latitudes worldwide (Decostere et al. 1999a, 1999b; Triyanto and Wakabayashi 1999; Figueiredo et al. 2005; Suomalainen et al. 2005; Schneck and Caslake 2006; Olivares-Fuster et al. 2007), including channel catfish, *Ictalurus punctatus* (Siluriformes: Ictaluridae), and zebrafish (or "zebra danio"), *Danio rerio* (Cypriniformes: Cyprinidae). Columnaris disease can decimate finfish populations in both recirculating and pond-based aquaculture operations and is one of two principal pathogens constraining the US farm-raised catfish industry (Plumb 1999). The disease is frequently diagnosed by the presence of a so-called "saddleback lesion," a symmetrical band of body discoloration flanking the dorsal fin (Cone et al. 1980). The histopathological attributes of saddleback lesions have been documented extensively and among many diseased finfish species in a variety of culture systems (Pate and Ordal

[1] Corresponding author.

1967; Cone et al. 1980; Morrison et al. 1981; Speare and Mirsalimi 1992; Kondo et al. 2002). The lesion itself can manifest as a rapidly spreading area of depigmentation and results in shallow ulceration (Cone et al. 1980). Surprising to us is that few ultrastructural observations of saddleback lesions exist and that none have been published previously for channel catfish or zebrafish. The majority of previous ultrastructural studies that document pathological changes to fish tissue associated with *Flavobacterium* spp. have focused on bacterial gill disease; especially acute and chronic changes to gill epidermal and chloride cells, gill lamellae, and gill filaments (e.g., Wakabayashi 1980; Kudo and Kimura 1983a, 1983b, 1983c, 1984; Foscarini 1989; Speare et al. 1991a, 1991b).

Herein, we use scanning electron microscopy (SEM) to illustrate the pathological features of experimentally induced, acute-stage columnaris disease, and associated saddleback lesions on the skin of laboratory-reared channel catfish and zebrafish.

Materials and Methods

Bacterial Immersion Challenge

Specific pathogen-free (SPF) channel catfish fingerlings of unknown sex ($n = 180$; mean weight = 4.2 g) were obtained from the School of Veterinary Medicine (Auburn University), stocked at 15 fish per each of three 37 L aquaria, acclimatized for 5 d before immersion bath, and fed daily to satiation with AQUAMAX Grower 400 (Purina Mills, Inc., St. Louis, MO, USA). SPF adult zebrafish of unknown sex ($n = 360$; mean weight = 0.6 g) were purchased from Aquatica Tropicals (Plant City, FL, USA), stocked at 30 fish per each of three 37 L aquaria, acclimatized for 5 d before immersion bath, and fed daily to satiation with 41% crude protein flakes (Central Garden and Pet, Franklin, WI, USA). Ten randomly selected individuals of each fish species were examined and proved culture negative for *F. columnare*.

Each aquarium had an individual biofilter and air stone. Water was checked daily to maintain established parameters (80 ppm alkalinity, 40 ppm hardness, 0.1 ppt salinity, 27 ± 1 C, pH 7.8 ± 0.2 [mean ± SE], ammonia, and nitrites non-detectable) and prepared with 340 g of Marine Salt (Seachem, Madison, GA, USA) diluted in 10 L of deionized water to make the primary salt stock. For tank use, 0.97 g of $CaCO_3$, 2.26 g of $NaHCO_3$, and 110 mL of the stock were mixed overnight in 55 L of deionized water.

Stock suspensions of the Japanese Collection of Microorganisms (JCM) 21327 isolate of *F. columnare* were stored in 10% glycerol at −80 C and routinely cultured in modified Shieh broth (Shoemaker et al. 2005) for 24 h at 26 C with gentle shaking. The immersion challenge was carried out by transferring fish (3×15 channel catfish or 3×30 zebrafish) to 15 L aerated buckets with 4 L of the same fresh water defined above and 40 mL of an overnight culture of the bacterium at approximately 5×10^6 colony forming units (CFU)/mL or 40 mL of modified Shieh broth for the controls. After a 0.5-h challenge, fish were divided equally among three aquaria and observed for saddleback lesions.

Scanning Electron Microscopy

Skin samples for SEM (Zeiss EVO 50 Variable Pressure Scanning Electron Microscope, Jena, Germany) were taken at 24 h post-challenge from control fish and from fish exhibiting a saddleback lesion. Fish were euthanized by overexposure (300 ppm) to the anesthetic MS-222 (Sigma, St. Louis, MO, USA) and observed to exhibit no gill movement for 10 min before immersion in 10% neutral buffered formalin for 48 h. Skin samples approximately 5 mm^2 were excised from both the margin and center of the observed saddleback lesions plus from those sites (normal) in control fish, gradually dehydrated in a graded series of ethanols using an automated tissue processor, transferred from 100% EtOH to a 50:50 (v:v) of 100% EtOH and hexamethyldisilazane (HMDS) for 1 h, transferred to and left in HMDS for 1 h, removed from HMDS and air-dried for 3 h, mounted on metal stubs using

FIGURE 1. *Arrows indicate anterior and posterior margins of "saddleback lesion" in channel catfish,* Ictalurus punctatus, *24 h post-exposure by immersion challenge with* Flavobacterium columnare *Japanese Collection of Microorganisms 21327 strain.*

two-sided sticky tape, and sputter coated with 15 nm of gold palladium.

Results

Observed grossly, control channel catfish had an uninterrupted pattern of uniformly gray skin (Fig. 1). Viewed with SEM, the surface epithelium was comprised of a continuous sheet of evenly distributed polygonal cells with fingerprint-like, well-delineated microridges (Figs. 1–11). Epithelial cells were closely apposed, lacking evidence of separation or gaps between these cells (Figs. 2–4) (Grizzle and Rogers 1976; Morrison et al. 1981; Ferguson 2006), with the exception of mucus pores that were often present at the confluence of three epidermal cells (Fig. 3). Control zebrafish exhibited comparable ultrastructural features, with the addition of scales that were arranged in a regular overlapping pattern within the dermis (Figs. 8, 9).

The saddleback lesions observed in channel catfish 24 h post-exposure to immersion challenge with *F. columnare* (JCM 21327 strain) were typical for columnaris disease (Cone et al. 1980). These lesions grossly comprised a pale, bilaterally symmetrical band of body discoloration beneath the dorsal fin extending anteriad to the pectoral fin origin and posteriad to the pelvic fin origin while wrapping ventrally around the belly (Fig. 1). The margin of the saddleback lesion was similar but particularly well-marked in zebrafish (Fig. 7), which had abundant and variously colored chromatophores along the body flanks. The expanding margin of the saddleback lesion

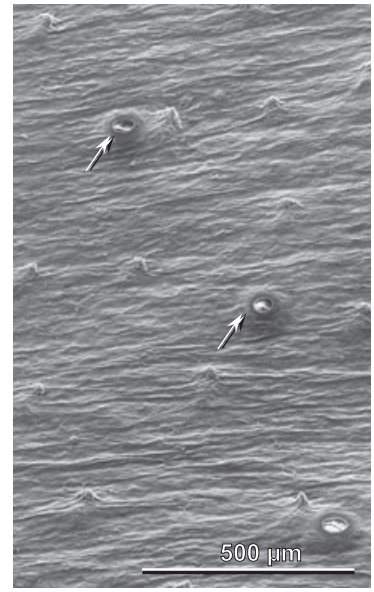

FIGURE 2. *Normal skin of channel catfish,* Ictalurus punctatus, *not exposed to* Flavobacterium columnare. *Note that skin comprises an even-surfaced, regular layer of epidermal cells, and lateral line pores (arrows) exhibit the typical architecture.*

in channel catfish (Fig. 5) harbored abundant rod-shaped bacterial cells, approximately 3–10 µm long × 0.3–0.5 µm wide, and was characterized by epidermal exfoliation and obliteration of epidermal cells. Cellular debris likely representing necrotic or swollen epidermal cells was evident in areas of the saddleback lesion and was heavily infiltrated by rod-shaped bacterial cells between and beneath the epidermal debris (Figs. 5, 6). No extravascular erythrocyte was observed in these areas, consistent with the lack of hemorrhage observed

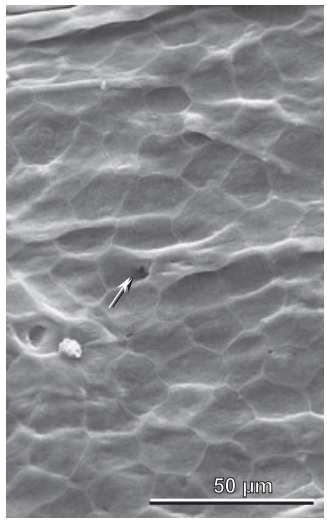

FIGURE 3. *Normal skin of channel catfish,* Ictalurus punctatus, *not exposed to* Flavobacterium columnare. *Epidermal cells are regularly distributed in epidermis and have closely apposed margins. Probable mucus pore (arrow). Cellular debris is largely absent from the field of view.*

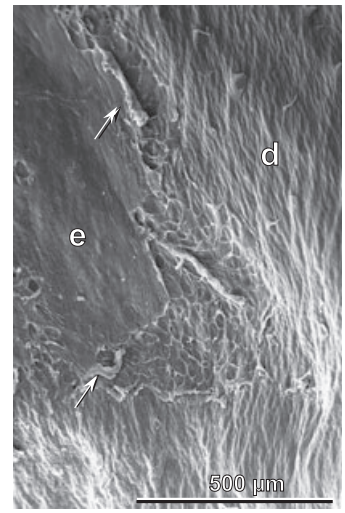

FIGURE 5. *Arrows indicate anterior margin of "saddleback lesion" in channel catfish,* Ictalurus punctatus, *24 h post-exposure by immersion challenge with* Flavobacterium columnare*, Japanese Collection of Microorganisms 21327 strain. Epidermis (e) is sloughing and dermis (d) is exposed.*

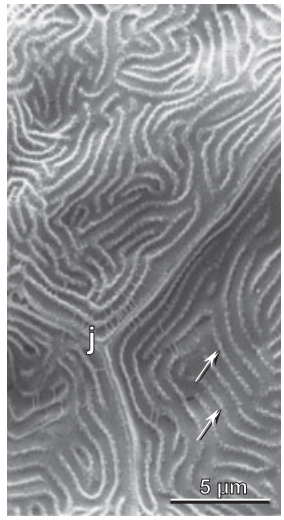

FIGURE 4. *Normal skin of channel catfish,* Ictalurus punctatus, *not exposed to* Flavobacterium columnare. *Epidermal microridges (arrows) appear normal and epidermal cell margins are closely apposed (j) and are intact.*

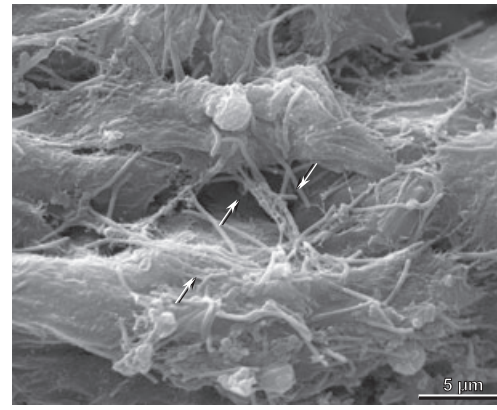

FIGURE 6. *A field within the "saddleback lesion" in channel catfish,* Ictalurus punctatus, *24 h post-exposure by immersion challenge with* Flavobacterium columnare, *Japanese Collection of Microorganisms 21327 strain. The wrinkled and uneven dermis has been denuded of overlying epidermis and a multitude of rod-shaped bacterial cells (arrows) and cellular debris, perhaps including necrotic epidermal cells, is present throughout the field of view.*

grossly on necropsy (Fig. 1). The underlying, exposed dermis beneath this cellular debris was rugose (Fig. 6). Saddleback lesions in zebrafish (Fig. 7) showed marked exfoliation of epidermal cells concomitant with abundant rod-shaped bacterial cells intermingled with cellular debris (Figs. 10, 11). Scales in lesioned

FIGURE 7. *Arrows indicate anterior and posterior margins of "saddleback lesion" in zebrafish,* Danio rerio*, 24 h post-exposure by immersion challenge with* Flavobacterium columnare *Japanese Collection of Microorganisms 21327 strain.*

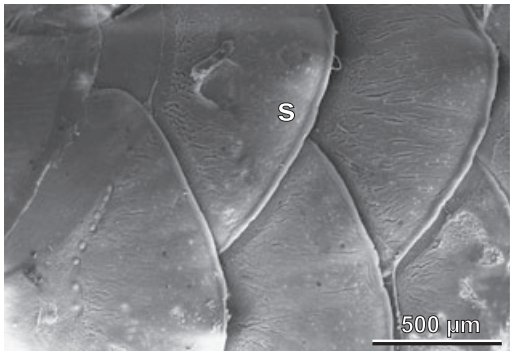

FIGURE 8. *Normal skin of zebrafish,* Danio rerio, *not exposed to* Flavobacterium columnare. *Note that epidermis covers each scale (s) and comprises an even- surfaced, regular layer of epidermal cells.*

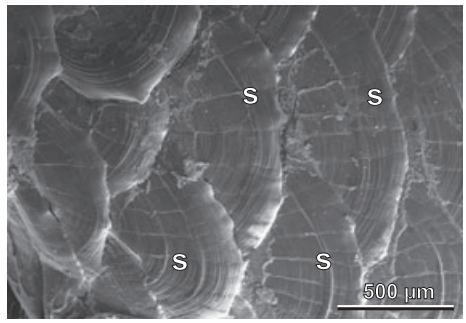

FIGURE 10. *A field within the "saddleback lesion" in zebrafish,* Danio rerio, *24 h post-exposure by immersion challenge with* Flavobacterium columnare *Japanese Collection of Microorganisms 21327 strain. Scales (s) are denuded of epidermis.*

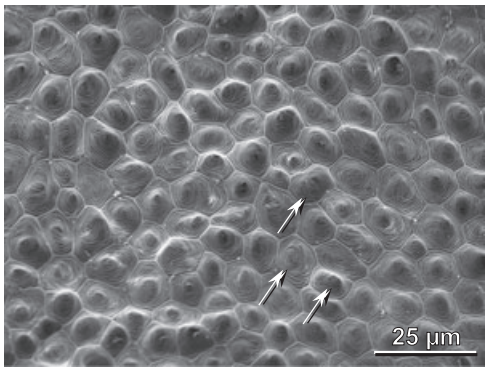

FIGURE 9. *Normal skin of zebrafish,* Danio rerio, *not exposed to* Flavobacterium columnare. *Epidermal cells (arrows) and their microridges are intact, and epidermal cell margins are closely apposed. The field of view is free of epidermal debris.*

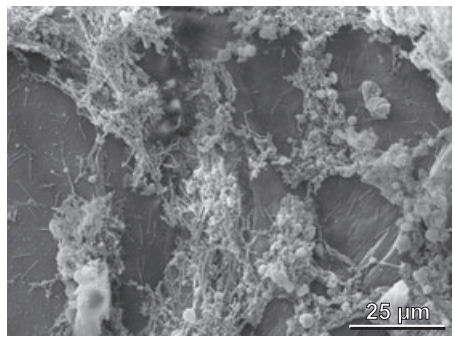

FIGURE 11. *A field within the "saddleback lesion" in zebrafish,* Danio rerio, *that lacks scales 24 h post-exposure by immersion challenge with* Flavobacterium columnare *Japanese Collection of Microorganisms 21327 strain. Numerous rod-shaped bacteria and cellular debris are distributed over the surface of the denuded dermis. Scales are absent from the field of view.*

areas were missing or, when present, denuded of epidermis, as evidenced by the ridged surface features of the cycloid scales themselves being evident with SEM (Fig. 10).

Discussion

The results of this study comprise the first published ultrastructural details of the surface features of the columnaris disease-induced

ulcerative dermatitis known commonly as saddleback lesions in channel catfish or zebrafish. These observations complement and generally agree with previous histopathological studies of skin associated with columnaris disease. Cone et al. (1980) detailed the histopathology of saddleback lesion in hatchery-reared underyearling Atlantic salmon, *Salmo salar*. The lesions were characterized by epidermal sloughing and necrosis in the stratum compactum, connective tissue, and somatic musculature. An abundance of bacterial cells generally corresponded to a damaged or obliterated epidermis, and bacterial cells infiltrated the dermis as well as between and within muscle fibers that were typically destroyed in their presence. Lesioned fish were sluggish and died 24 h after exhibiting the saddleback lesion. Morrison et al. (1981) and Speare and Mirsalimi (1992) reported pathological changes associated with saddleback lesions that are comparable with Cone et al. (1980) and this study: SEM of the saddleback lesion margin in Atlantic salmon showed a marked transition from a uniform, normal epidermis to a rugose surface that had epidermal cell remnants covering the exposed dermis. Scale loss was evident in some areas of the lesion. Likewise, Kondo et al. (2002) studied bacterial cold water disease on the body surface of ayu, *Plecoglossus altivelus*, associated with *Flavobacterium psychrophilum* in Japan. Their SEM micrographs showed exfoliation of epidermal cells, loss of microridge structure, and epidermal hyperplasia. Our observations differ from those of Kondo et al. (2002) in that we did not observe obvious epithelial hyperplasia in lesioned areas (Figs. 6, 11).

Epidermal cell exfoliation is herein observed as an acute response to *F. columnare* (JCM 21327 strain) exposure in channel catfish (Figs. 1, 5, 6) and zebrafish (Figs. 7, 10, 11). In freshwater fishes, epidermal damage leads to osmotic imbalance by water infiltrating exposed tissues, a process commonly known as "waterlogging" (Ferguson 2006). From our current results, it can be inferred that channel catfish and zebrafish suffer osmotic imbalance resulting from the documented epidermal damages we observed with SEM. Although we cannot confirm it with SEM, we speculate that the pale lesions observed grossly in channel catfish and zebrafish (Figs. 1, 7) resulted from loss of the underlying layer of pigmented cells as clearly the tegument of the fish was compromised. The severity of this pathological change complements our observation that, under our infection model conditions, *F. columnare* JCM 21327 causes cumulative mortalities of up to 100% in channel catfish and up to 85% in zebrafish (Arias, unpublished data).

Routine SEM preparations do not stabilize or preserve the mucus layer of fishes, but the interaction between bacterial pathogens and fish mucus deserves further study because mucus is the first fish component that bacteria contact during colonization or adhesion (Speare and Mirsalimi 1992; Martínez et al. 2004). Speare and Mirsalimi (1992) specified the "local" and "mucus flow" models for understanding how mucigen granules and other components of the mucus laminate the external surface of the fish, thereby protecting it from pathogens. We find it noteworthy that the mucus flow model, which predicts that mucins are secreted into and replenish a dynamic mucus coat that passively flows over the fish's body, may help explain the pattern of saddleback lesion development in the channel catfish and zebrafish we studied herein. If bacterial cells reside in or on the mucus cuticle, which seems highly likely given that *F. columnare* cells adhere to skin (Olivares-Fuster et al. in press), these bacteria would be translocated from anterior to posterior, provided bacterial cell gliding motility is negligible. Albeit perhaps coincidentally, this matches reports (Pate and Ordal 1967; Cone et al. 1980) that the saddleback lesion advances posteriorly from the dorsal fin.

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